PRESENTED
TO
THE FACULTY OF SCIENCE
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY.


Thesis submitted for Degree of Ph.D.

Degree of Ph. D. conferred

28th June, 1928.

# THE COLLOIDAL PROPERTIES OF HUMAN SERUM WITH SPECIAL REFERENCE TO THE CHANGES THAT TAKE PLACE IN SYPHILIS.

A Thesis for the degree of Ph.D.Edinburgh

by

Cecil I.B.Voge B.Sc.

(From the research laboratory of the Royal College of Physicians . Edinburgh . )

---

It is well known that as a result of syphilitic infection various changes occur in blood sera, and that for the detection of these changes several reactions have been elaborated some of which are of great clinical importance. Of these the complement fixation test commonly known as the Wassermann reaction is of particular significance.This test which need not be described in detail depends on the fact that when an antigen consisting of an alcoholic extract of tissue particularly heart or liver added to physiological saline solution,is mixed with syph-:ilitic serum containing complement,part or all of the complement is fixed or inactivated in such a way that it is unable to effect haemolysis of sensitised red blood cells.

EX BIBL. UNIV. EDINBURGEN

When serum from a non-syphilitic individual is substituted in the place of syphilitic serum little or no fixation of complement takes place. The antigen used in the Wassermann reaction is a colloidal suspension containing negatively charged particles and at an early date the suggestion was made that the primary change that occurs when syphilitic serum is used in the Wassermann reaction is a decrease in the degree of dispersion of the colloidal particles of the antigen. Evidence in favour of this view has been advanced in several directions. In particular it has been found that antigens of the same type as those used in the Wassermann reaction when added under certain conditions to sera from cases of syphilis give a precipitate visible to the naked eye, whereas under the same conditions no precipitation occurs when normal sera are used.. On the basis of this observation methods have been devised for the detection of syphilis in the human subject, and of these the methods of Sachs and Georgi (1), Dryer (2), and Kahn (3), may be mentioned.

That these tests lead to results which are in substantial agreement with those obtained by the Wassermann reaction seems to be generally acknowledged and so the view suggests itself that the Wassermann reaction depends essentially on some alteration in the colloidal condition of the antigen similar to that which clearly occurs

in the formation of a precipitate when these precipit-:ation tests are carried out.

There are however certain facts which appear to make the acceptance of this view difficult. Thus certain sera are met with which react positively in the Wass-:ermann test and negatively in the precipitation test, and other sera are found which react negatively to the former and positively to the latter. Notwithstanding these difficulties many workers are inclined to the view that some common factor is responsible for the occurrence of positive results in the two reactions. More generally it would appear legitimate to make the tentative assumption that for an explanation of the characteristic fixation of complement observed in the Wassermann reaction the colloidal properties of the serum and of the antigen are of fundamental importance, and that some insight into the nature of this reaction might reasonably be expected to follow from an inves-:tigation into the colloidal properties of the serum.

Although from the point of view of theoret-:ical interest and of clinical importance the Wassermann reaction is preeminent amongst all tests for the det-:ection of syphilis in the human subject, the precipita-:tion tests are much simpler in nature and form a more suitable starting point from which to investigate the colloidal properties of serum in relation to the changes which occur in syphilis. As already mentioned these tests may be carried out by adding

to the serum under suitable conditions a colloidal suspension essentially similar to that used as antigen in the Wassermann reaction. Our knowledge of the constitution of such an antigen is however very incom-
:plete, and its colloidal properties are in many ways peculiar and puzzling.
Since it has been found that other colloidal suspensions may be substituted for these lecithin suspensions in carrying out the precipitation tests, and since these suspensions are more normal in their colloidal behaviour, it appears therefore desirable first of all to study in some detail the behaviour of these less complex suspen-
:sions when added to sera.

It is well known that the Wassermann ~~reaction~~ the precipitation tests may be applied not only to serum but also to the cerebro spinal fluid. It is in fact in relation to the cerebro spinal fluid that the use of the simpler type of antigen has met with the greatest success. The colloidal gold reaction of Lange is in common use for the clinical diagnosis of certain forms of cerebro spinal syphilis, and more recently the mastic reaction of Emmanuel and the colloidal benzoin reaction of Guillain, Laroche, and Lechelle have been widely applied for the same purpose. A large number of other reactions of the same general type have also been suggested and appear to yield more or less satisfactory results. In the cerebro spinal fluid the total amount of protein present is usually quite small and for this reason

it is easier to investigate the nature of these precip-:itation tests with the cerebro spinal fluid than it is in the case of serum which contains much larger quantities of protein.

It appears therefore that of the reactions cap-:able of destinguishing a normal body fluid from one which has been altered as the result of syphilitic infection, one of the least complex is that between cerebro spinal fluid and one of these comparatively simple negatively charged colloidal suspensions. It is natural therefore that such a reaction should have been chosen as a starting point in the investigation of these serological phenomena, and it is necessary at this point to give a brief account of the work which has been carried out in this laboratory in this direction. The colloidal gum benzoin reaction was chosen for investigation, and examination was made of certain factors which influence the occurrence of precipitation. In order to explain the results arrived at it is necessary first of all to describe some of the experimental find-:ings, and also to state certain generally accepted principles in colloidal chemistry which are of funda-:mental importance for the present investigation .

The colloidal gum benzoin test of Guillain, Laroche and Lechelle is carried out by making a series of dilutions of the cerebro spinal fluid with a 0.01 per cent solution of sodium chloride. The first tube contains 0.75c.c. cerebro spinal fluid and 0.25c.c. sodium chloride solution. The second, 0.5c.c. cerebro spinal fluid and 0.5 c.c. sodium chloride solution . The third 0.25 c.c. cerebro spinal fluid and 0.75 c.c. sodium chloride solution. The fourth 0.125 c.c. cerebro spinal fluid and 0.875 c.c. sodium chloride solution, and each succeeding tube contains half the amount of cerebro spinal fluid contained in the preceeding one. Sodium chloride is always present in such a quantity that the final volume is 1 c.c.. To each tube 1c.c. of colloidal gum benzoin is added. The preparation of this suspension is described in the experimental part of the present thesis.
The degree of precipitation which occurs in the various tubes is observed after 12 to 24 hours . With a normal fluid no precipitation occurs in the first five tubes but complete or partial precipitation may occur in tubes 6 and 7.. If however cerebro spinal fluid from a patient suffering from general paralysis of the insane is used a characteristic precipitation occurs in at least some of the first five tubes and in the case of a fluid giving a strongly positive complete precipitation may occur in all of the five tubes.
Although this procedure here described for the carrying out of the reaction is very convenient from a practical

point of view, it is not adapted for theoretical inves-
:tigations. A complication which it is necessary first
of all to eliminate lies in the fact that when the test
is carried out in the above manner, not only does the
concentration of fluid vary from tube to tube but the
hydrogen ion concentration of the final mixture also
varies. It is known particularly from the researches
of Loeb that the colloidal properties of proteins are
very strongly influenced by the hydrogen ion concen-
:tration. It was for this reason that it was found
necessary to carry out at the same time and on the
same fluid a large number of observations so that the
effects of the alteration of the hydrogen ion concen-
:tration and of the concentration of the fluid proteins
could be separated from each other. In order to do
this most conveniently a series of rows of tubes was
prepared instead of a single row. To each tube of any
particular row a definite amount of acid or alkali
was added. The corresponding tubes in different rows
thus contained the same concentration of fluid but the
hydrogen ion concentration differed from tube to tube.
In order most readily to interpret the results it was
found convenient to represent them by means of a graph
in which the ordinates represented the ~~the~~ $P_h$ and the
abscissae the concentrations of the fluid. On this
graph points were marked corresponding to tubes in
which complete or almost complete precipitation

occurred. The graph which was obtained in the case of normal cerebro spinal fluid is shown in fig.1.

It will be observed from this graph that a region exists such that complete or almost complete precipitation occurs in any tube if the $P_h$ and cerebro spinal fluid concentration are represented by a point within this region. This region is shaded in fig.1 and will be called the region of normal precipitation. If the point representing a tube lies without this region no precipitation or slight precipitation is observed. If the point lies in the area marked (+) the colloidal particles are found to bear a positive electrical charge, that is to say under the influence of an electric field they migrate towards the negative pole, and when the point lies within the area marked (-) the particles in the corresponding tube bear a negative charge. It appears that the region of precipitation separates the region of positive charge from the region of negative charge. It is further observed that the region of positive charge and the region of precipitation do not extend to a higher $P_h$ than 4.7.

In order to explain the phenomenon summarised by this graph it is necessary to state briefly some general principles of the colloidal chemistry of suspensions and of proteins. In the cases of suspensions of small particles such as gold and gum benzoin the stability depends on the existence of an electrical

Fig. 1

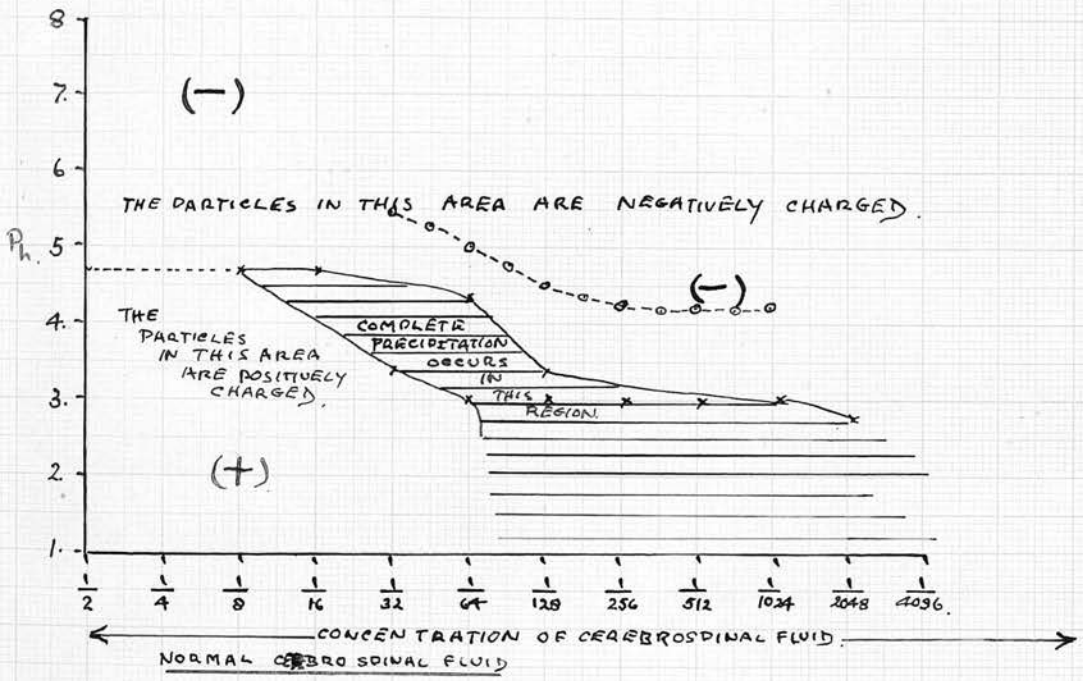
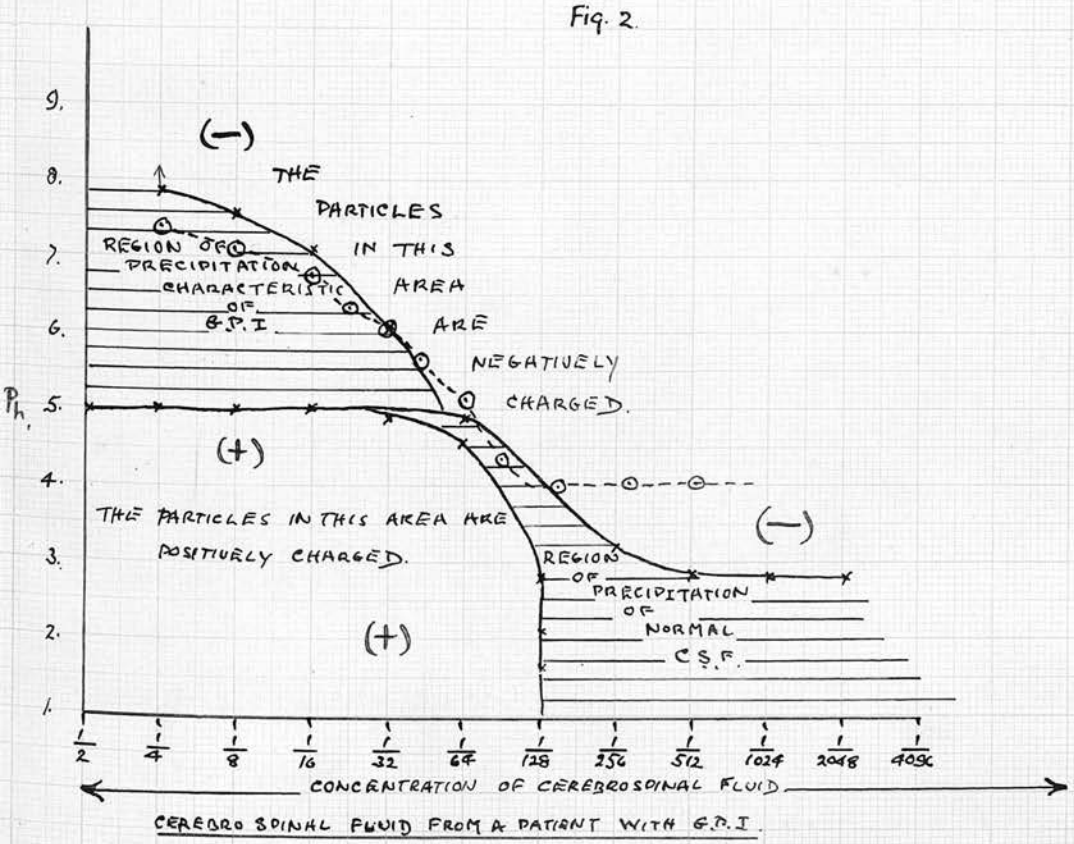

8
7
6
5
4
3
2
1
Ph.
(−)
THE PARTICLES IN THIS AREA ARE NEGATIVELY CHARGED.
(−)
THE PARTICLES IN THIS AREA ARE POSITIVELY CHARGED.
COMPLETE PRECIPITATION OCCURS IN THIS REGION.
(+)
1/2 1/4 1/8 1/16 1/32 1/64 1/128 1/256 1/512 1/1024 1/2048 1/4096
CONCENTRATION OF CEREBROSPINAL FLUID
NORMAL CEREBRO SPINAL FLUID

Fig. 2.


9.
8.
7.
6.
5.
4.
3.
2.
1.
Ph.
(−)
THE PARTICLES IN THIS AREA ARE NEGATIVELY CHARGED.
REGION OF PRECIPITATION CHARACTERISTIC OF G.P.I
(+)
THE PARTICLES IN THIS AREA ARE POSITIVELY CHARGED.
(−)
REGION OF PRECIPITATION OF NORMAL C.S.F.
(+)
1/2 1/4 1/8 1/16 1/32 1/64 1/128 1/256 1/512 1/1024 1/2048 1/4096
CONCENTRATION OF CEREBROSPINAL FLUID
CEREBRO SPINAL FLUID FROM A PATIENT WITH G.P.I.

potential difference between the particles and the surr-:ounding medium. This is commonly expressed by saying that the particles must be electrically charged. In consequence of this charge they repel each other when they approach closely and thus they continue to remain separate. The suspension therefore does not flocculate, if the particles ~~if the particles~~ were originally so small that their Brownian movements are sufficiently great to overcome the action of gravity. When however the electrical charge is neutralised the surface forces normally act in such a way that they cause the particles to adhere if they approach sufficiently close to one another. Under these conditions instead of remaining separate they form aggregates which sediment under the action of gravity.

When a sufficient quantity of electrolyte is added to a colloidal suspension of the above type flocculat-:ion usually occurs. For convenience ~~the discussion~~ the discussion will be limited to the case in which the colloidal particles are originally negatively charged. The cations of the added electrolyte are the important factor in determining the effect. Since these cations are positively charged they are able to neutralise the negative charge on the colloidal particles, and ~~so to~~ so to effect flocculation. The higher the valency of the cations the more effectively do they neutralise the charges on the particles and so the more effectively do they bring about precipitation.

In the case of tervalent cations, not only is the negative charge on the colloidal particle neutralised, but under certain conditions a positive charge is conferred upon them as a result of which the suspension is rendered stable and does not precipitate. This phenomenon has been investigated by Kermack and Voge, (4-) using methods similar to ~~that~~ those employed in the present investigation.

It is well known however that the precipitation of negatively charged colloidal suspensions may be effected not only by inorganic cations but also and even more effectively by organic cations. For instance, aniline hydrochloride precipitates more effectively than sodium chloride and the precipitating power of certain basic organic dyestuffs is particularly pronounced. The markedly precipitating power of organic cations appears to be related to the tendency of these cations to be adsorbed on surfaces. It is clear that if the cations are rapidly adsorbed on the surface of the particles the charge on the particles will be completely neutralised even at low concentrations of these organic cations.

A class of organic cations of especial importance in relation to the present investigation is that which includes the cations derived from proteins in the presence of an acid . It is generally recognised that proteins behave as ampholytes, that is to say in the presence of a base such as sodium hydroxide they

function as an acid and form a salt, the organic part of which is negatively charged, whilst in the presence of an acid such as hydrochloric acid they function as a base and form a salt the organic part of which is positively charged. At a certain $P_h$ the protein as a whole is found to possess no aggregate charge. This $P_h$ is called the isoelectric point of the protein. At any higher $P_h$ the protein is negatively charged and at any lower $P_h$ it is positively charged. In the case of the common proteins of the serum and the cerebro spinal fluid the isoelectric point lies from 4.7 to 5.52. It follows therefore that at a $P_h$ less than 4.7 any protein which is present will exist as a cation, and will bear a positive charge. Further, proteins, the molecules of which are large, are readily adsorbed on surfaces and it is therefore to be expected that the precipitating action of a protein at a $P_h$ less than 4.7 will be marked. It is also to be expected that protein solutions will not be able to effect precipitation at a $P_h$ greater than 5.5.. It will be seen from the graph that these expectations are in fact realised. Further the existence of the region of positive charge in the graph indicates that the positively charged protein is adsorbed on the surface of the colloidal particles to such an extent that they acquire a positive charge and thus are rendered stable. The region of precipitation corresponds to conditions under which positively charged protein reduces the negative charge on the particles but does

not confer on them a positive charge sufficiently great to ensure stability.

When the cerebro spinal fluid of a patient suffering from general paralysis of the insane is studied by the above method a graph such as that represented in fig.(2), is finally obtained. It is seen at once that the principle difference between the two graphs is that in the second one there is a region of precipitation which is absent in fig. (1). This region of precipitation lies between a $P_h$ 5 and $P_h$ 8 , and does not exist in concentrations of fluid less than 1/64. Apart from this region the two graphs agree closely. In both of them there is a region in which the particles are positively charged and one in which they are negatively charged. These regions are separated by a region of precipitation.

It may be noted here that in both diagrams a line --o---o-- appears. This line passes through points which correspond to the $P_h$ and concentrations of fluid present in the single row of tubes to which neither acid nor alkali was added. Its particular form is determined by the acidity of the gum benzoin suspension, and the buffering substances present in the cerebro spinal fluid. The gum benzoin suspension is slightly acid having a $P_h$ of 4.2 and so a mixture of it with distilled water, or unbuffered salt solution, or cerebro spinal fluid in high dilution, is acid and has a $P_h$

less than 4.7. If however it is added to undiluted cerebro spinal fluid the sodium bicarbonate and the buffering substances in the fluid neutralise the acid of the gum benzoin and the $P_h$ of the resulting mixture is about 8. The line thus starts at a $P_h$ of about 8 and follows the course represented in the graph dropping below a $P_h$ of 5 and finally running almost horizontally at a $P_h$ 4.0. It will be seen that it ~~runs through~~ approaches the region of normal precipitation at a point corresponding to the 6th, or 7th, tubes in the single row of dilutions containing neither acid nor alkali, and it becomes clear why precipitation often occurs in these two tubes. Slight variation in the protein content of the cerebro spinal fluids or in the amount of buffering salts which it contains will change the relative position of this line and the zone of precipitation and so in cases of normal fluid in particular no precipitation may occur in the 6th. or 7th tubes. On the other hand in fluids from cases of general paralysis of the insane in which the total protein content is usually increased the region of normal precipitation tends to occur at a some-:what higher dilution than in the case of normal fluids, and so marked precipitation usually occurs in the 6th, and 7 th tubes as well as in some or all of the first five tubes.

In order to account for the region of normal precipitation which occurs in the graph relating to

fluid from a case of general paralysis of the insane, it was suggested by Wright and Kermack (5) that such a fluid might contain a protein with a high isoelectric point. Such a protein would exist as a cation at any $P_h$ below the isoelectric point and so might effect precipitation of the negatively charged gum benzoin particles at any $P_h$ below the isoelectric point. It is in order to investigate the validity of this hypothesis that a considerable part of the work described in the present thesis has been carried out.

The above work on the cerebro spinal fluid has been described somewhat fully because the investigation in the present thesis forms a continuation of it. The question naturally arises as to whether regions of precipitation similar to those met with in the case of cerebro spinal fluids will be found when blood serum is investigated in a similar way. If analogous regions are found the further question arises as to which serum fractions are responsible for the various regions, and in particular for any regions of abnormal precipitation characteristic of serum from a person suffering from a syphilitic infection. In order to ascertain whether the precipitation phenomena characteristic of syphilis might in fact be accounted for by the presence of a protein of high isoelectric point the relatively accessible protamine Clupeine, (isoelectric point $P_h$12-) has been prepared, and normal serum containing small

quantities of this protamine has been examined by a method similar to that described above, so that the results might be compared with those obtained in the case of serum from syphilitic individuals.

The work has been extended by ascertaining the effect of the addition of clupeine to the various protein fractions from normal serum and it will be found that in general the effect of the addition of this protamine to normal serum or to its protein fractions causes them closely to simulate serum, or the respective serum fractions, derived from a syphilitic subject.

It should be emphaised that clupeine has been used in the present investigation because, of all the protamines, it is perhapsthe most readily accessible and the best known. There is therefore no suggestion that clupeine itself is in anyway responsible for the precipitation phenomena characteristic of syphilis. It is clear that the similarities which have been observed between normal serum containing clupeine and serum of syphilitic individuals might be accident-:al and might possess no real significance. Evidence that the resemblances are not altogether superficial has therefore been sought for by investigating the behaviour in the Wassermann reaction of normal serum containing clupeine. If such serum effects the fixation of complement it appears legitimate to consider this as some indication that a compound at least of the

same general type as clupeine may actually be respons-
:ible for the characteristic changes which appear in
the body fluids as the result of syphilitic infection.
The final section of the experimental part of the pres-
:ent thesis therefore contains an account of the applic-
:ation of the Wassermann reaction to normal serum
containing small quantities of clupeine.

## EXPERIMENTAL

### 1. General.

The general method used in the experiments which form the major portion of the present investigation has already been described in the introduction. It is however convenient to give a fuller account here of the details of the procedure. In the first place the glassware used was cleansed throughly by treatment with sulphuric acid - bichromate mixture, followed by washing first with tap water and then with distilled water.

In order to carry out a set of observations the foll-:owing procedure was adopted. As a concrete example the examination of normal serum will be described.

A series of dilutions of serum, hydrochloric acid and sodium hydroxide were prepared. To each of a series of tubes were added 0.5 c.c. of serum of various strengths, 0.5c.c of the appropriate acid or alkali solution and finally 1c.c. of gum benzoin sol.

This colloidal solution was prepared as described in the appendix. The results are conveniently summarised in table 1 in which the marginal figures represent the concentrations of acid or alkali and of serum in the mixture after the gum benzoin has been added. The concentrations originally prepared were four times those recorded.

The tubes were observed after they had stood at room temperature for 24 hours and readings were made accord-:ing to the following convention.

| | | |
|---|---|---|
| Complete precipitation | = 4 = | Clear supernatant fluid. |
| Almost complete | = 3 = | A slight haze in the supernatant fluid. |
| Half precipitated | = 2 | |
| Hardly any precipitate | = 1 = | The particles of the suspension have become aggregated but are not precip-:itated. |
| No precipitation | = 0 = | Quite opalescent. |

In the case of those tubes in which precipitation had occurred the hydrogen ion concentration was approx-:imately estimated by the addition of a drop of suitable indicator solution to about 0.5c.c. of the supernatant fluid. The tint developed was compared with those represented in the chart published by Clark, and it was found that, with some practice, read-:ings could be obtained which were consistent within 0.2 $P_h$ units. In many cases, however, it has been of interest to determine the $P_h$ of the tubes where precipitation had not occurred. Owing to the whiteness of the gum benzoin sol a certain amount of difficulty was encountered but this was surmounted by a compari-:son with known colour standards and the results were found to fit in with the values determined in the ad-:jacent zones of precipitation.

The areas of protection were determined by adding 1c.c.

of a 4% sodium chloride solution to all tubes where precipitation had not occurred, this amount and concentration being more than enough to precipitate the original gum benzoin sol. After 12 hours readings are made as follows:-

No change = Complete protection
Half precipitation = Half protection
Complete " " " = No protection.

The charges upon the gum benzoin particles were determined for the most part by an ultramicroscopic examination, where the drift of the particles was observed under the influence of an electric field, as described by Wright and Kermack (5).
A more systematic investigation using an inverted U tube has been carried out in some cases.

As mentioned in the introduction it is convenient to represent the results in a graph, in which the ordinates represent the $P_h$ values and the abscissae represent the concentration of the serum . In addition to the regions of precipitation and of non precipitation it is often convenient to indicate also the region which is such that the contents of a tube, represented by any point of it, exhibits the phenomenon of protection, that is to say: the colloidal particles are not precipitated by a concentration of salt solution amply sufficient to cause precipitation of the unprotected gum benzoin sol.

The significance of these regions of protection will be referred to in the general discussion.

It must be noted that on account of the limits of experimental accuracy the boundaries of the various regions are only approximately correct.

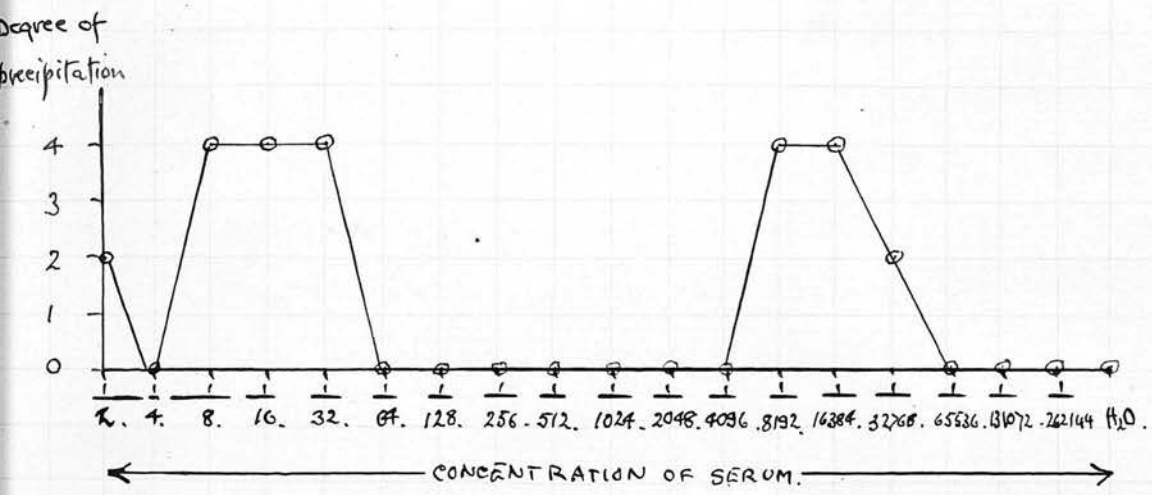
2.Examination of normal serum and normal serum fractions

a)Normal serum.

When a series of dilutions of normal serum in distilled water is prepared, and an equal volume of gum benzoin sol is added to each, the following result shown in fig. (3) is obtained. The ordinates represent the degrees of precipitation and the abscissae the concentration of serum after the gum benzoin has been added. There is very little in the first tube and no precipitation in the second. In these two tubes the gum benzoin particles are negatively charged and complete protection exists. Precipitation now occurs and continues to be observed till a concentration of 1/128 is reached when a zone of non precipitation is again found. Throughout this zone the particles are positively charged and with the exception of the first tube there is no protection. A small zone of precipitation follows at about 1/8192 and 1/16384 and this is again followed by non precip-:itation which continues till the last tube is reached which contains no serum but only distilled water. The particles in this latter zone are negatively charged and are not protected.

It may be noted at this point that if the serum be heated at 55°C before being submitted to this test the degree of precipitation which is observed in the first few tubes is less, and if the heating has been continued for 30 mins no precipitation at all was

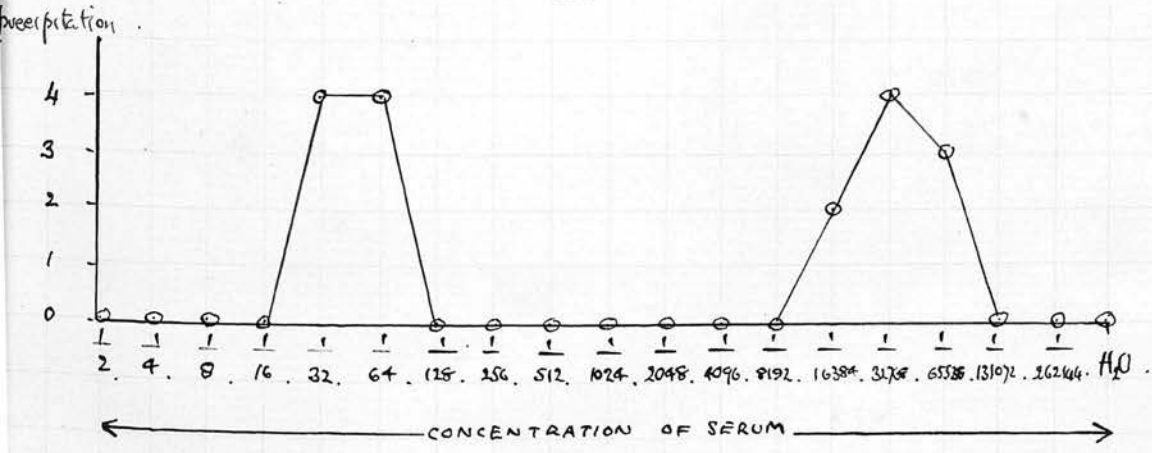
Fig 3.




NORMAL SERUM

UNHEATED

Fig 4.




NORMAL SERUM

HEATED 30 MINS AT 55°C.

found to occurr in the first four tubes.
The results of a test carried out on a serum so heated are shown in fig.(4). It is seen that compared with fig.(3),the areas of precipitation and protection are the same with the exception that the first zone of non precipitation is now much more pronounced. It will also be seen that the charges upon the particles are the same for the different zones as they were before.
As explained in the introduction an inspection of these figures affords very little real information from the theoretical point of view,particularly in view of the fact that the hydrogen ion concentration varies from tube to tube and the necessity therefore arises of examining systematically the effect of altering the Ph and the concentration of the serum independently of each other.
A complete series of tubes was therefore arranged and serum and acid and alkali in suitable dilutions added to each as described in the general section above. Colloidal gum benzoin was then added and the usual observations were made at the end of 24 hours. The results obtained are summarised in Table I and fig 5. The figure has been prepared according to the principles already set forth in the general section and the intro-:duction and need not therefore again be explained in detail. The serum used in this experiment was heated for 30 mins. at 55°C. before the dilutions were made.

TABLE 1

| CONCENTRATION ACID OR ALKALI. | ← CONCENTRATION OF SERUM → | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/2048 | 1/4096 | 1/8192 | 1/16384 | 1/32768 | 1/65536 | $H_2O$ |
| N/25 HCl | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 4 | 4 | 4 |
| 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 4 |
| 100 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 |
| 200 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 |
| 400 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 |
| 800 | 0 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 0 |
| 1600 | 0 | 0 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 0 |
| 3200 | 0 | 0 | 0 | 3 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 |
| 6400 | 0 | 0 | 0 | 4 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 |
| $H_2O$ | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 0 | 0 | 2 | 4 | 4 | 0 | 0 | 0 |
| 6400 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 |
| 3200 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1600 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 800 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 400 NaOH | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

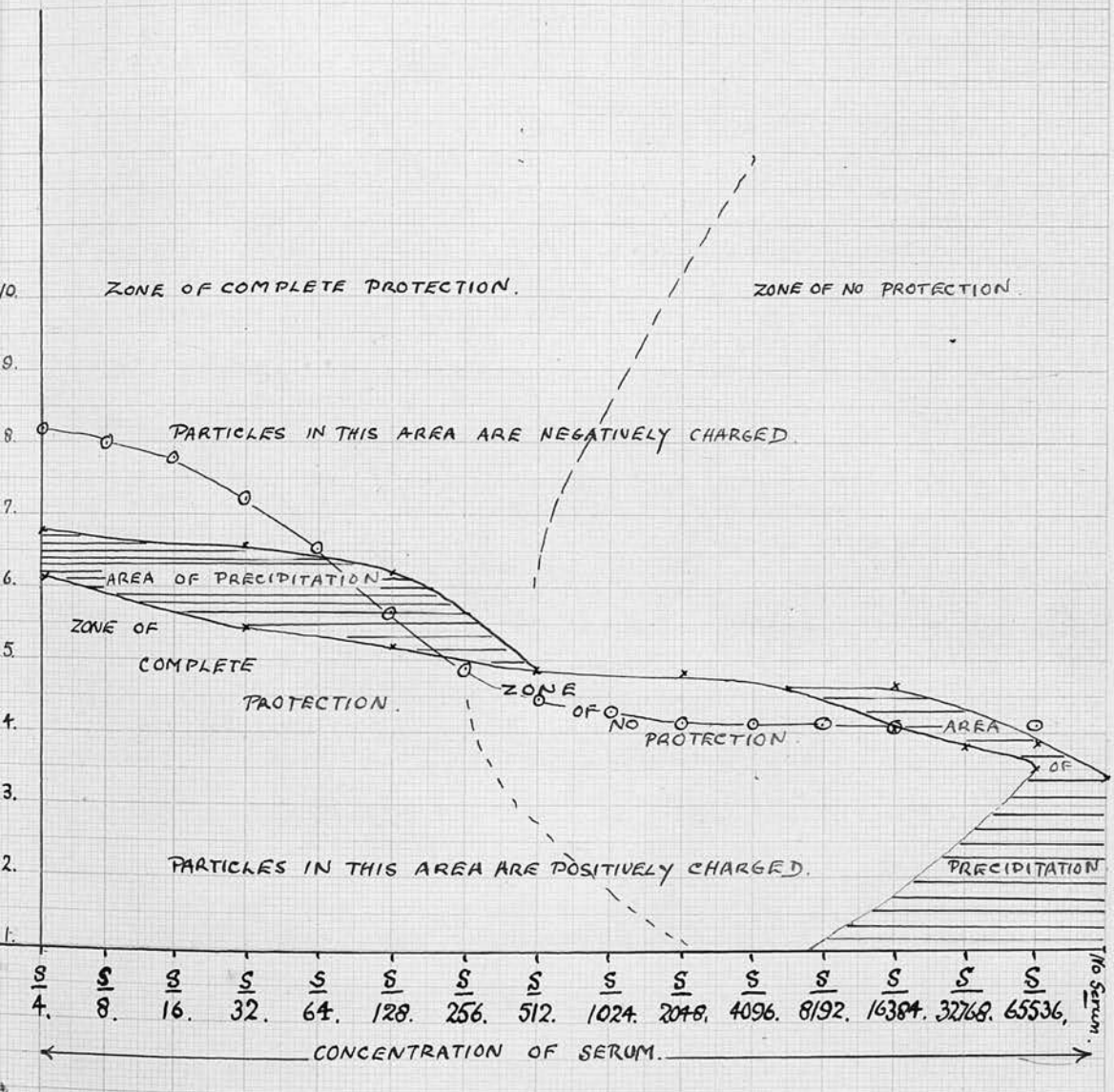
NORMAL SERUM and COLLOIDAL GUM BENZOIN.

SERUM HEATED AT 55°C FOR 30 MINS BEFORE EXPERIMENT.

COMPLETE PROTECTION DENOTED 0"

HALF PROTECTION DENOTED 0'

Fig 5.




THE $P_h$ VALUES OF EACH TUBE IN THE ROW CONTAINING NO ACID OR ALKALI. ⊙——⊙

NORMAL SERUM.

THE SERUM USED IN THIS EXPERIMENT WAS HEATED FOR 30 MINS AT 55°C.

It will be observed from fig.( 5 ) that a region of precipitation exists which is very similar and obviously analogous to that which is found in fig.(1) which refers to cerbro spinal fluid. This region however occurs at a concentration of serum considerably greater than the concentration of cerbro spinal fluid at which the region occurs in fig.(1). This of course is the natural consequence of the fact that the concentration of protein is much greater than that in normal cerbro spinal fluid. This high concentration of protein in serum is probably also related to the marked broadening of the region of precipitation which is observed in fig.( 5 ) at relatively high serum concentrations. For instance at a concentration of 1/4 precipitation occurs between $P_h$s 6.2 - and 6.8. It should be remembered that serum protein is far from being homogeneous and that a small fraction of it probably possesses a higher isoelectric point than the average. Thus with high concentrations of protein there may be a sufficiently high concentration of positively charged molecules even at a Ph 6.5 to neutralise the charges on the gum benzoin particles and so bring about precipitation. . It is therefor not surprising that this broader zone should be found.

It is of particular interest to observe the course of the dotted line in fig.(5). It will be recalled that this line is such that any tube in figure (5), (to which neither acid nor alkali had been added),

is represented by a point on it. It will be seen that in consequence of the high concentration of serum protein, the region of precipitation is so far to the right in fig (5) that it cuts this line twice, and so in the single row of dilutions represented in fig.(3), two zones of precipitation are observed instead of the single one, (or none at all) observed with normal cerbro spinal fluid. It may also be noted that the particles in the non precipitated tubes between the two zones are positively charged, since the corresponding points in fig.(5) lie in the region of positive charge.

Under certain conditions, as when the serum is deficient in protein, the relative positions of the dotted line and the region of precipitation may be such that the line does not cross but enters it and emerges again on the same side. In this case only one zone of precipit-:ation is observed, and only one zone of precipitation has in fact occasionally been found when single rows of dilutions of serum are examined.

It may further be observed on reference to fig.(5) that all the unprecipitated particles up to a conc-:entration of 1/256 are fully protected. At lower concentrations no protection exists.

Table 2.

| CONCENTRATION OF NaCl. | ← CONCENTRATION OF SERUM → 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/2048 | 1/4096 | 1/8192 | 1/16384 | $H_2O$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 12% | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 |
| 6% | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 3% | 0 | 0 | 0 | 0 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 1·5% | 4 | 3 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 0·75% | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 0·37% | 0 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 |
| 0·18% | 0 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 |
| 0·09% | 0 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 3 | 4 | 4 | 4 |
| 0·04% | 0 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 0 |
| $H_2O$. | 0 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 0 |

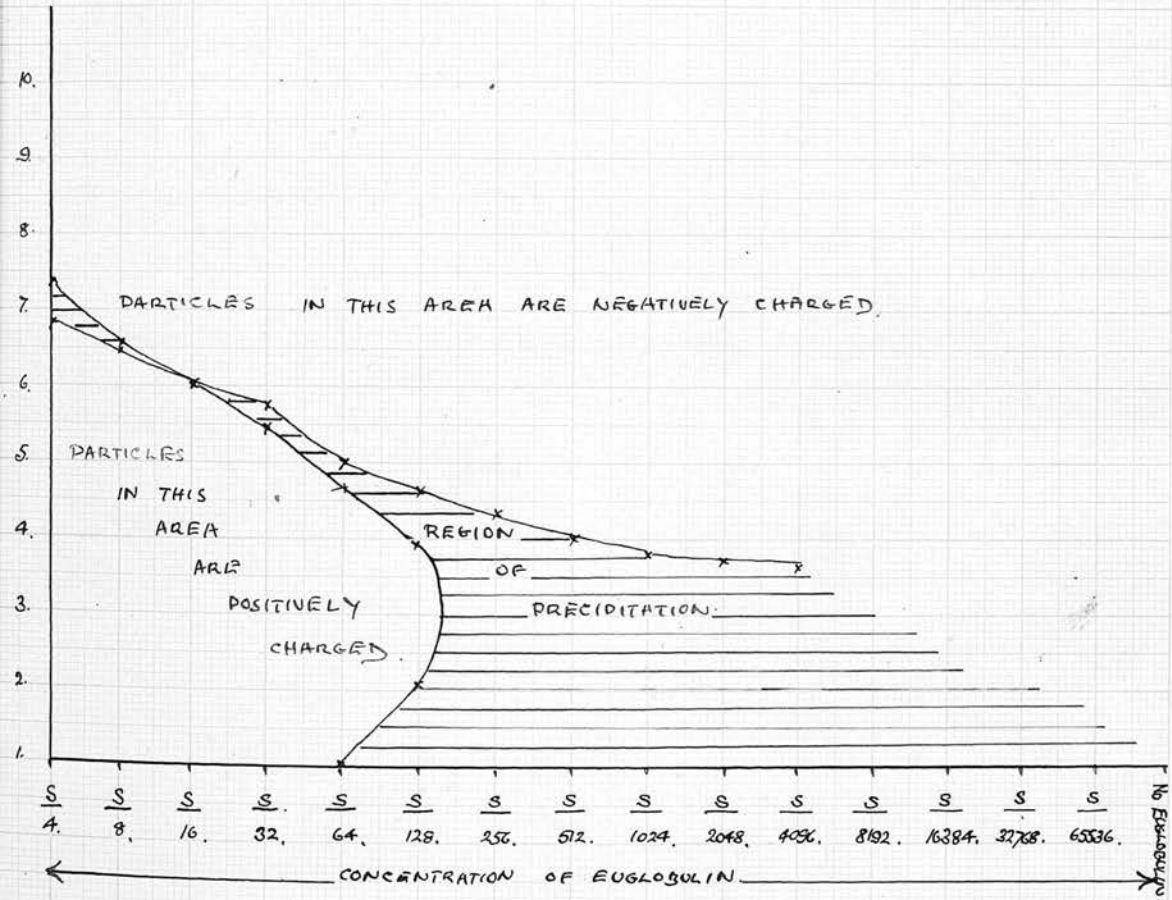
NORMAL SERUM.

UNHEATED.

It is of some interest to observe the effect of the presence of neutral inorganic electrolytes on the precipitation of gum benzoin by normal serum. This is particularly desirable as by this means it is possible to obtain some idea of the action exerted by hydro-:chloric acid and sodium hydroxide, in virtue of their acting as inorganic electrolytes, and apart from their action in altering ~~their action in altering~~ the serum by confering on it a positive or negative charge. The experiment, the results of which are summarised in table 2, were therefore carried out. The details of this experiment which are exactly analogous to the experiments with acid and alkali will be readily gathered from the table. It will be apparent that as long as the sodium chloride concentration remains below 0.7% there is very little difference in the different rows. Between 0.7% and 1.5% the first zone of non precipitation disappears. When the salt concentration is greater than 1.5% the gum benzoin particles are protected and this protection becomes more marked as the salt concentration increases up to 12%. Above this value no results have been recorded.

It may be at once remarked that this table suffers from the same defect, from the point of view of theor-:etical investigation, as does the single row. The hydrogen ion concentration differs from tube to tube and in consequence it is difficult to draw positive conclusions.

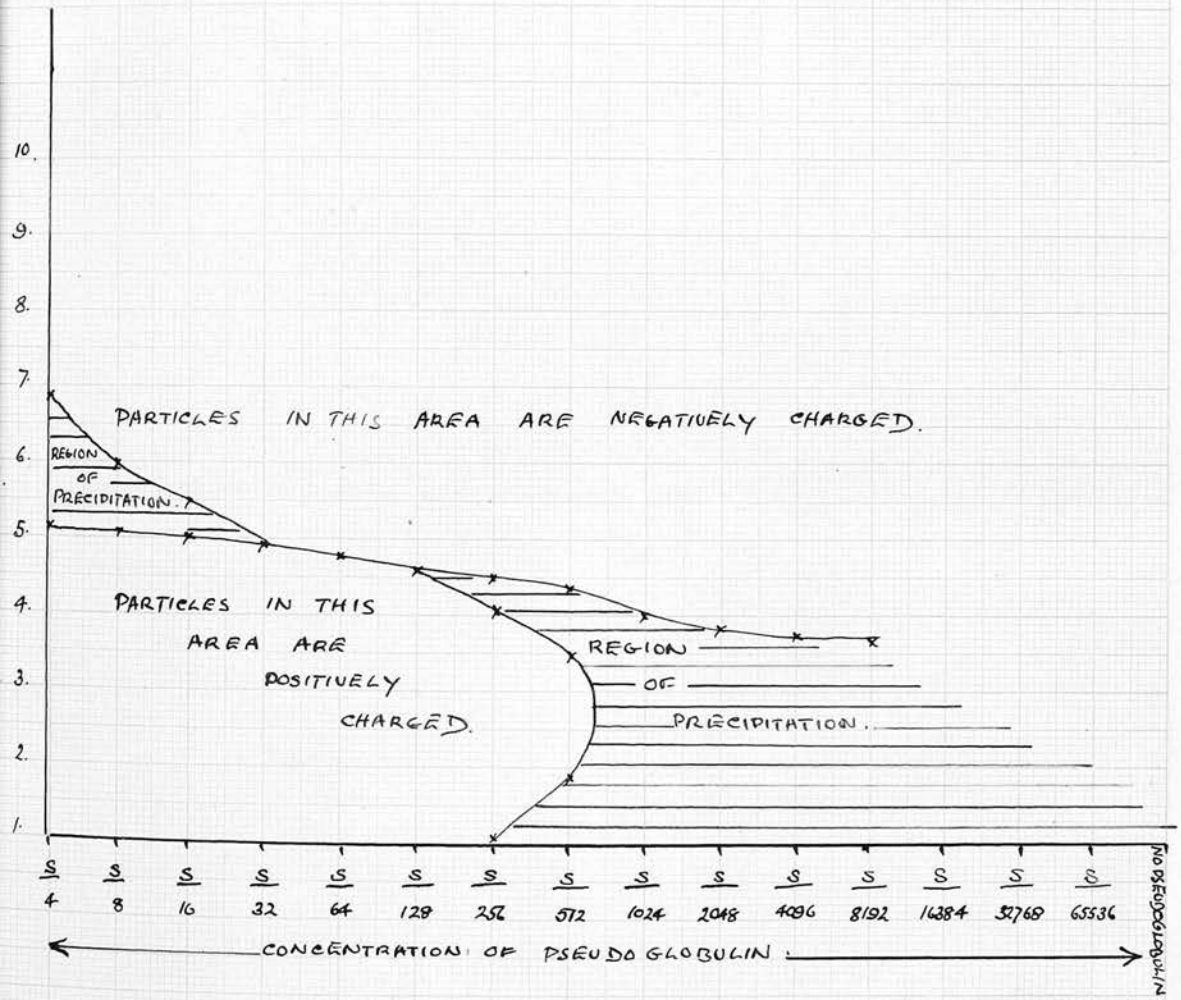
Fig. 6.




EUGLOBULIN OBTAINED FROM NORMAL SERUM.
THE EUGLOBULIN USED IN THIS EXPERIMENT WAS UNHEATED.

and the solid residue is placed

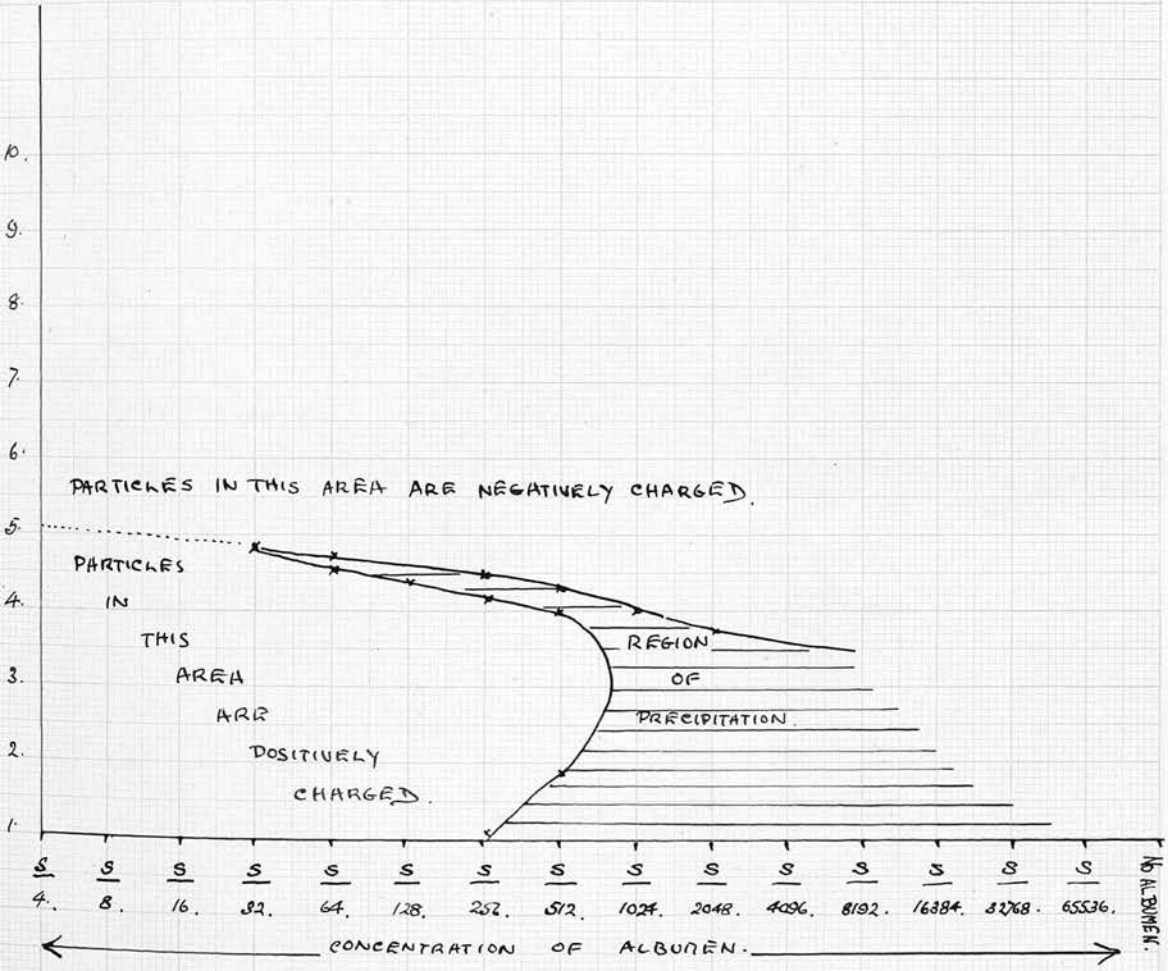
Fig. 7.




PSEUDOGLOBULIN OBTAINED FROM NORMAL SERUM.
THE PSEUDOGLOBULIN USED IN THIS EXPERIMENT WAS UNHEATED.

and the solid residue is placed

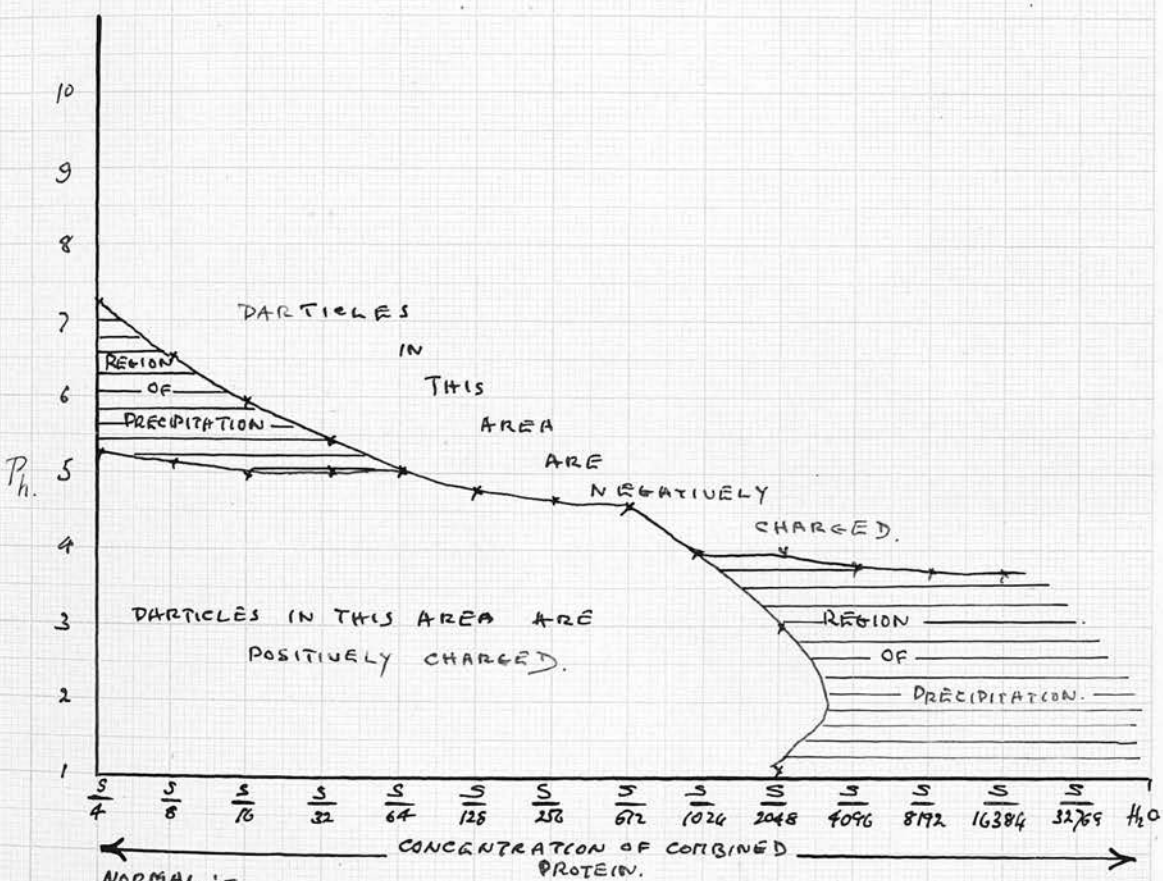
Fig. 8




ALBUMEN OBTAINED FROM NORMAL SERUM
THE ALBUMEN IN THIS EXPERIMENT WAS UNHEATED.

and the solid residue is placed

Fig. 4.




NORMAL :-

EUGLOBULIN
PSEUDOGLOBULIN
ALBUMEN
} THESE THREE FRACTIONS RECOMBINED.

ALL UNHEATED.

THIS IS VERY SIMILAR TO THE GRAPH OBTAINED WITH UNHEATED NORMAL SERUM.

It may however be concluded that sodium chloride exerts very little influence on the precipitation phenomena at concentrations lower than 0.7% or approximately .08 normal, whereas the effects of hydrochloric acid which we have been considering are obtained with concentrations below N/50. It is therefore reasonable to conclude that the hydrochloric acid and sodium hydroxide used in these experiments exerts it's action in virtue of the characteristic alteration it effects in the protein, a conclusion which is in accordance with the generally accepted views in the chemistry of proteins.

b) The protein fractions of normal serum.

In human blood serum at least three distinct protein fractions can be distinguished, euglobulin, pseudoglobulin, and albumen. Normal serum was therefore fractionated by the method described below and the fractions so obtained were examined in exactly the same way as was the whole serum. An experiment was also carried out with the three fractions recombined in order to ascertain whether this gave the same results as the original serum.

The method adopted to ensure complete separation of euglobulin was to precipitate both globulins with half saturated ammonium sulphate. The supernatant fluid which contains the albumen, is removed by filtration and the solid residue is placed

in a collodion sac and dialysed. Water passes in and the solid goes into solution. As dialysis continues the salt content decreases and the euglobulin commence s to come out of solution, complete separation being obtained only when the salt content is zero. The super-:natant fluid contains the pseudoglobulin and this is separated from the solid euglobulin by centrifuging. The albumen is precipitated by saturated ammonium sulphate, the solid mass is placed in a collodion sac and dialysed till salt free.

It was necessary to fit a mercury manometer to the collodion sac during dialysis as otherwise the volume of liquid inside the sac becomes unduly large. When the manometer is present a back pressure is exercised which opposes the entrance of water into the sac and so prevents the volume increasing.

When solutions of pseudoglobulin and albumen have been obtained, they are made up with distilled water to the original volume of serum and inorganic salts were added such that the salt concentration would be that of serum. The euglobulin was made up to the original volume with Ringer's solution.

Thus three solutions were obtained each containing a single protein fraction, but identical with the original serum in volume and in content of inorganic salts.

The results obtained when these three fractions were examined according to the general method described

above are represented in figs. (6,7,8,9.)
It will be observed that in each diagram there occurs a region of precipitation analogous to that we have called the region of normal precipitation when dealing with the cerbro spinal fluid. In the case of the albumen solution this is the only region of precipitation which occurs. In the cases of euglobulin and pseudoglobulin however the region of precipitation extends so as to occur at high concentrations of the protein solutions just as it does in the case of normal serum. This region however is not so broad in the cases of these single proteins, but in the case of pseudoglobulin it occurs at a somewhat lower Ph than it does with euglobulin. It is therefor to be expected that when all proteins are present in solution together as they are in normal serum or in the solution used in the experiment with the recombined fractions from which fig.( 9 ) was obtained, the region of precipitation would be broad and well marked.

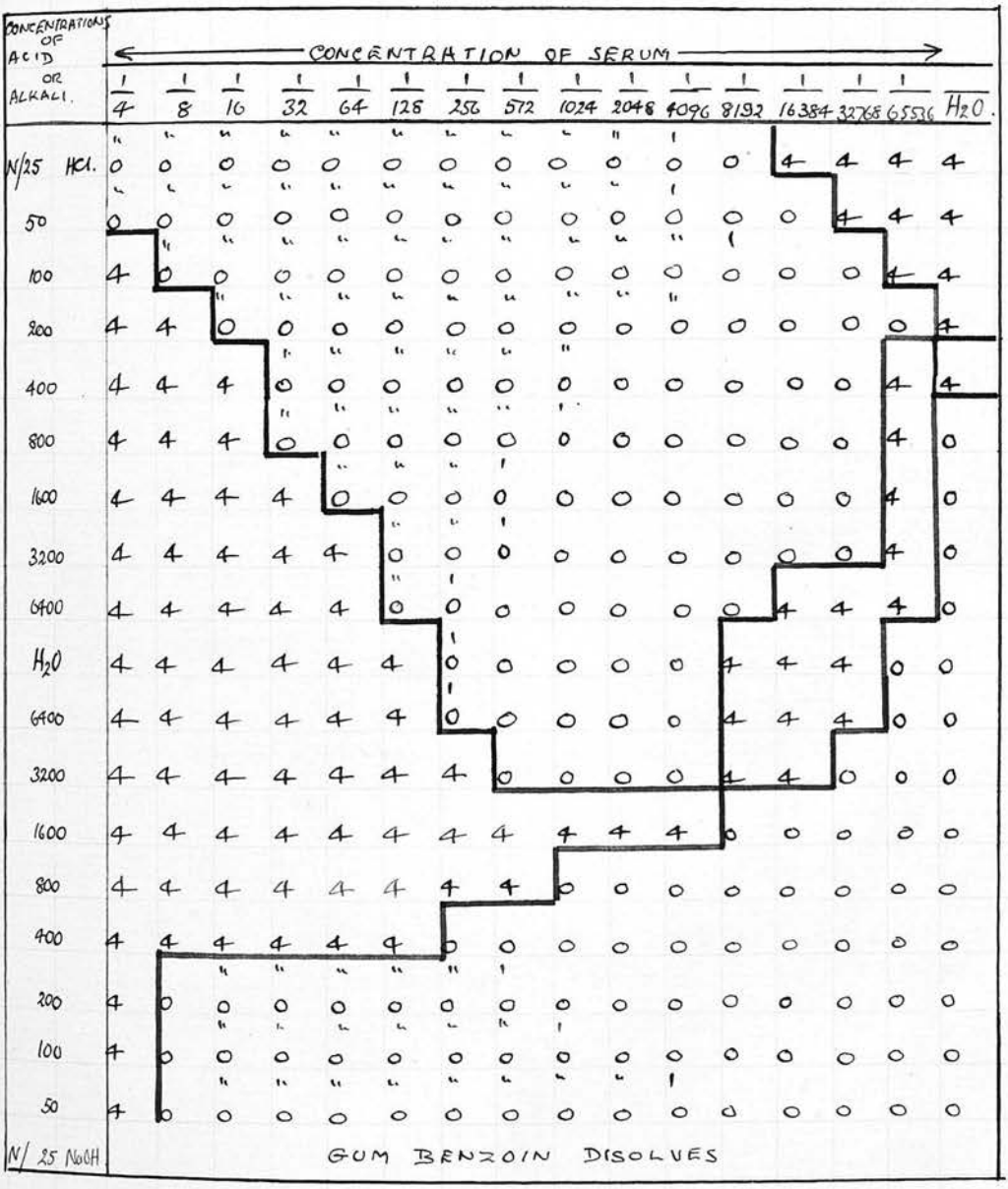
Reference may be made here to a phenomenon which occurs when experiments such as the above are carried out. When preparing a series of mixtures of serum with acid or alkali opalescence is observed in certain tubes before the gum benzoin has been added. In fig.(9a) are shown the $P_h$ values of each tube before the addition of gum benzoin. The area of opalescence is outlined in black and it may be noted that this corresponds with the region of precipitation which is observed after the gum benzoin is added in as far as the opalescence occurs between $P_h$ 5 and $P_h$ 7.

Fig. 9a.

| HCl | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/2048 | $H_2O$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CONCENTRATION OF SERUM | | | | | | | | | | | |
| N/40 | 6.0 | 4.8 | 4.6 | 4.4 | 3.2 | 3.0 | 2.8 | 2.4 | 2.2 | 1.8 | 1.6 | 1.2 |
| 80 | 6.2 | 6.0 | 5.0 | 4.6 | 4.0 | 3.8 | 3.6 | 3.4 | 3.0 | 2.8 | 2.4 | 2.0 |
| 160 | 6.4 | 6.2 | 5.4 | 5.0 | 5.0 | 4.6 | 4.4 | 4.0 | 3.8 | 3.6 | 3.4 | 2.4 |
| 320 | 7.0 | 6.8 | 6.4 | 5.6 | 5.0 | 4.8 | 4.2 | 4.0 | 3.8 | 3.7 | 3.6 | 2.8 |
| 640 | 7.4 | 7.2 | 6.8 | 6.4 | 5.2 | 5.0 | 4.8 | 4.4 | 4.2 | 4.0 | 3.6 | 3.4 |
| 2560 | 7.8 | 7.4 | 7.2 | 7.0 | 6.6 | 5.8 | 5.0 | 4.8 | 4.6 | 4.2 | 4.0 | 3.6 |
| 5120 | 8.0 | 7.8 | 7.6 | 7.4 | 6.8 | 6.6 | 5.8 | 5.4 | 5.0 | 5.0 | 5.0 | 4.8 |
| $H_2O$ | 8.2 | 8.0 | 7.8 | 7.6 | 7.0 | 6.6 | 6.2 | 6.0 | 6.0 | 6.0 | 6.0 | 6.0 |
| 400 | 8.6 | 8.6 | 8.6 | 8.6 | 8.6 | 8.6 | 8.6 | 8.6 | 8.6 | 8.6 | 8.6 | 8.6 |
| N/40 NaOH | : | : | : | greater than $P_h$ 11 | | | | | : | : | : | : |

AREA OF OPALESCENCE OUTINED IN BLACK.

Table 3.




SYPHILITIC SERUM.

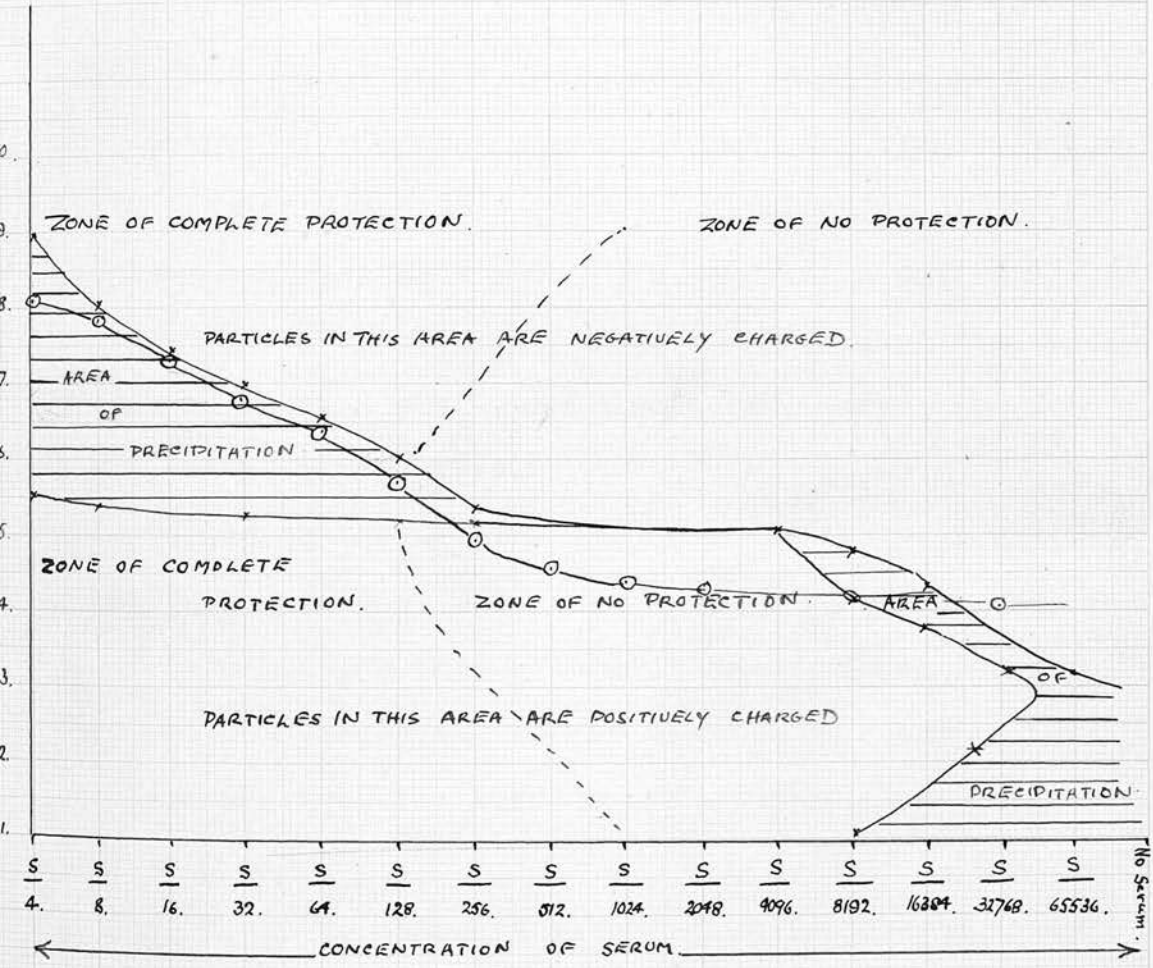
HEATED 30 MINS AT 55°C.

[THE SAME PRECIPITATION WOULD BE OBTAINED WITH UNHEATED SERUM.]

COMPLETE PROTECTION DENOTED 0

HALF PROTECTION DENOTED 0'

Fig. 10




THE $P_h$ VALUES OF EACH TUBE IN THE ROW CONTAINING NO ACID OR ALKALI ⊙——⊙

SYPHILITIC SERUM.

THE SERUM USED IN THIS EXPERIMENT WAS HEATED FOR 30 MINS AT 55°C.

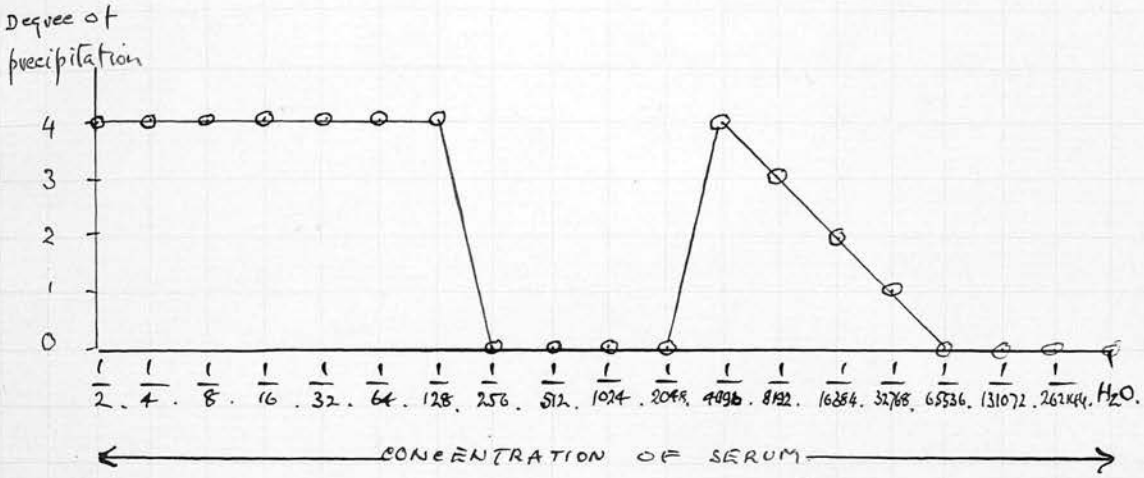
3. Examination of serum and serum fractions derived derived from a syphilitic subject.

So far we have studied the serum reactions given by serum from normal individuals, normal in the sense that they do not give a positive Wassermann or Sigma reaction. In the present section a corresponding series of observations has been made using the serum of a patient suffering from syphilis. Only serum which gave a markedly positive reaction when submitted to the Wassermann and Sigma tests was employed.

In addition to the examination of whole serum observations were also made with the three protein fractions as was done in the case of normal serum. These fractions were prepared according to the method described in the previous section. The results are summarised in figs. (12, 13 and 14.).

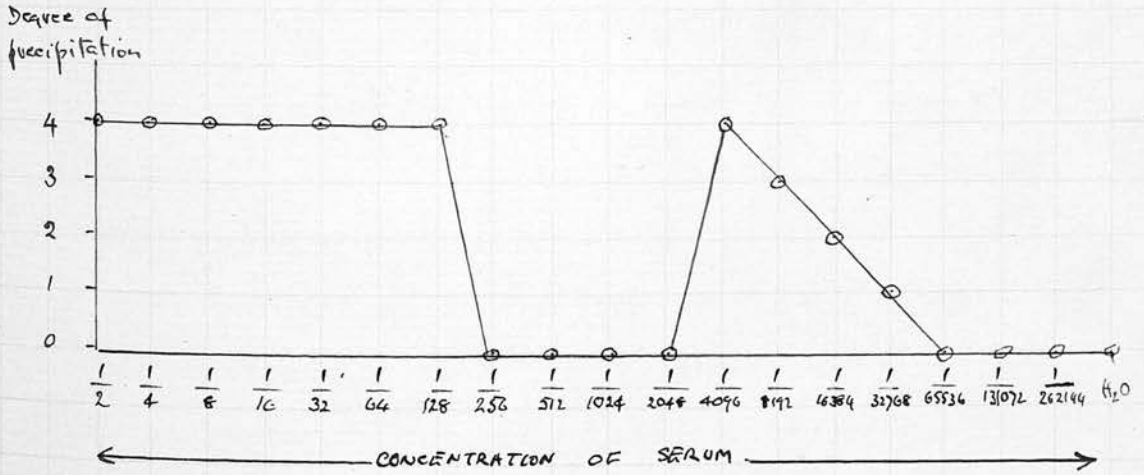
If fig. (10) is compared with fig. (5), (normal), both of which refer to sera heated for 30 mins at 55°C it will be seen that what has been called the normal region of precipitation is essentially similar in the two figures. One characteristic difference is apparent. In the case of normal serum even at relatively high concentrations no precipitation occurs above Ph 7. In the case of syphilitic serum precipitation occurs at Ph 8.5 - 9.0 so that the region which previously extended between a $P_h$ 5 and $P_h$ 7 is now more marked and broadened. It will at once be realised that this change is very similar to that observed when cerebro spinal fluid

Fig 11.




SYPHILITIC SERUM

UNHEATED.




SYPHILITIC SERUM.

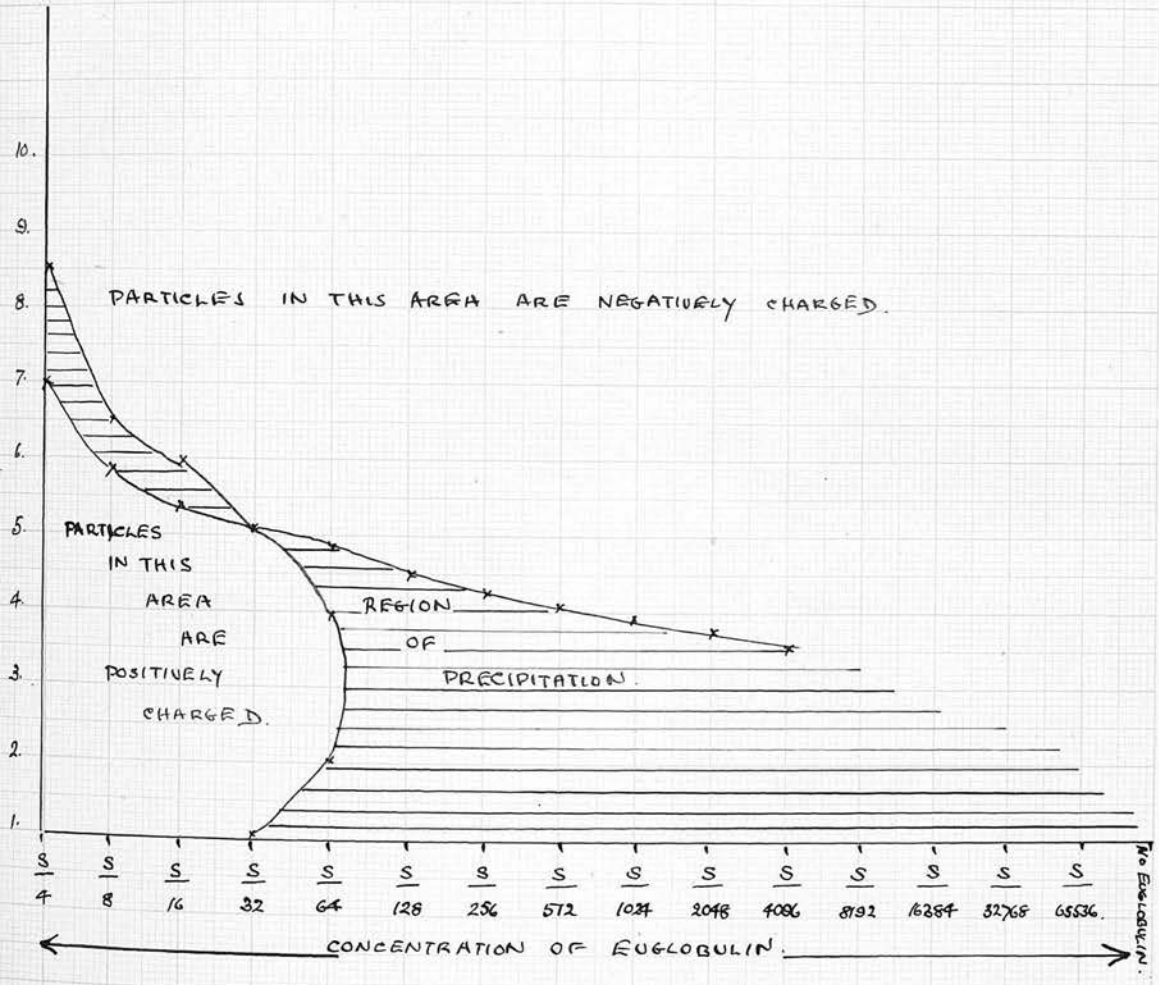
HEATED 30 MINS AT 55°C

from a case of general paralysis of the insane is compared with that from a normal individual. The result of a syphilitic infection is therefore closely parallel both in the cerebro spinal fluid and in the serum, as in both cases the power is developped of specially precipitating gum benzoin at a $P_h$ greater than that at which precipitation normally occurs.

It should here be mentioned that the exact position of the region of abnormal precipitation in the case of syphilitic serum is not altogether constant. This is not surprising if it be remembered that the concentrat-:ions of the individual protein constituents of the blood serum vary particularly in disease as well as the concentrations of certain inorganic salts.

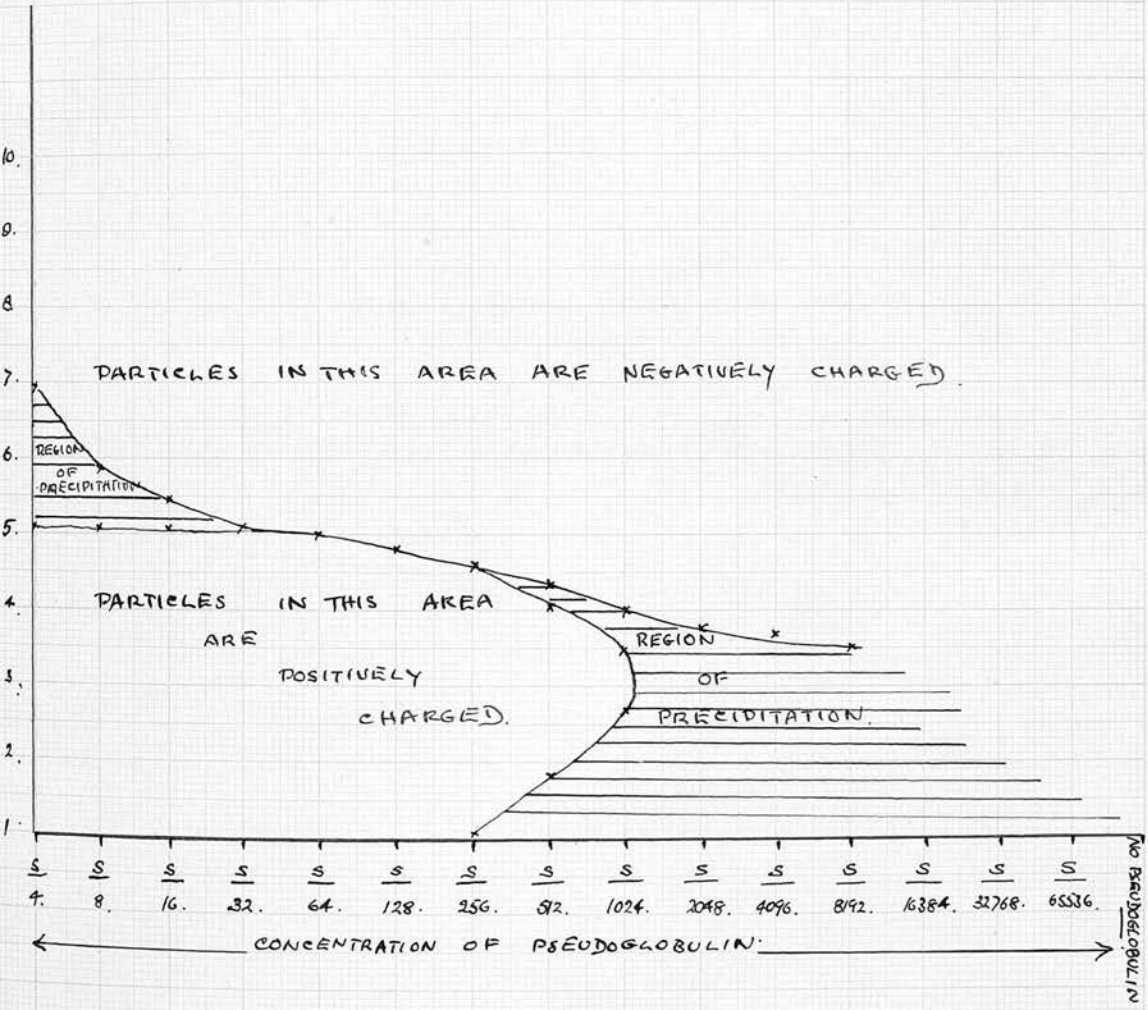
In fig. (10) the dotted line as before passes through points corresponding to a series of dilutions of serum to which neither acid nor alkali have been added. It will be seen that according to this figure this line at high concentrations of serum lies in the region of abnormal precipitation and this implies that when a series of dilutions of serum in distilled water is prepared and gum benzoin added precipitation should occur in the first few tubes. Evidence that this usually does take place has been adduced in a paper already published but it should be noted that the consistency of this result is not absolute and that certain seras from cases of syphilis do not cause precipitation in these tubes.

Fig. 12



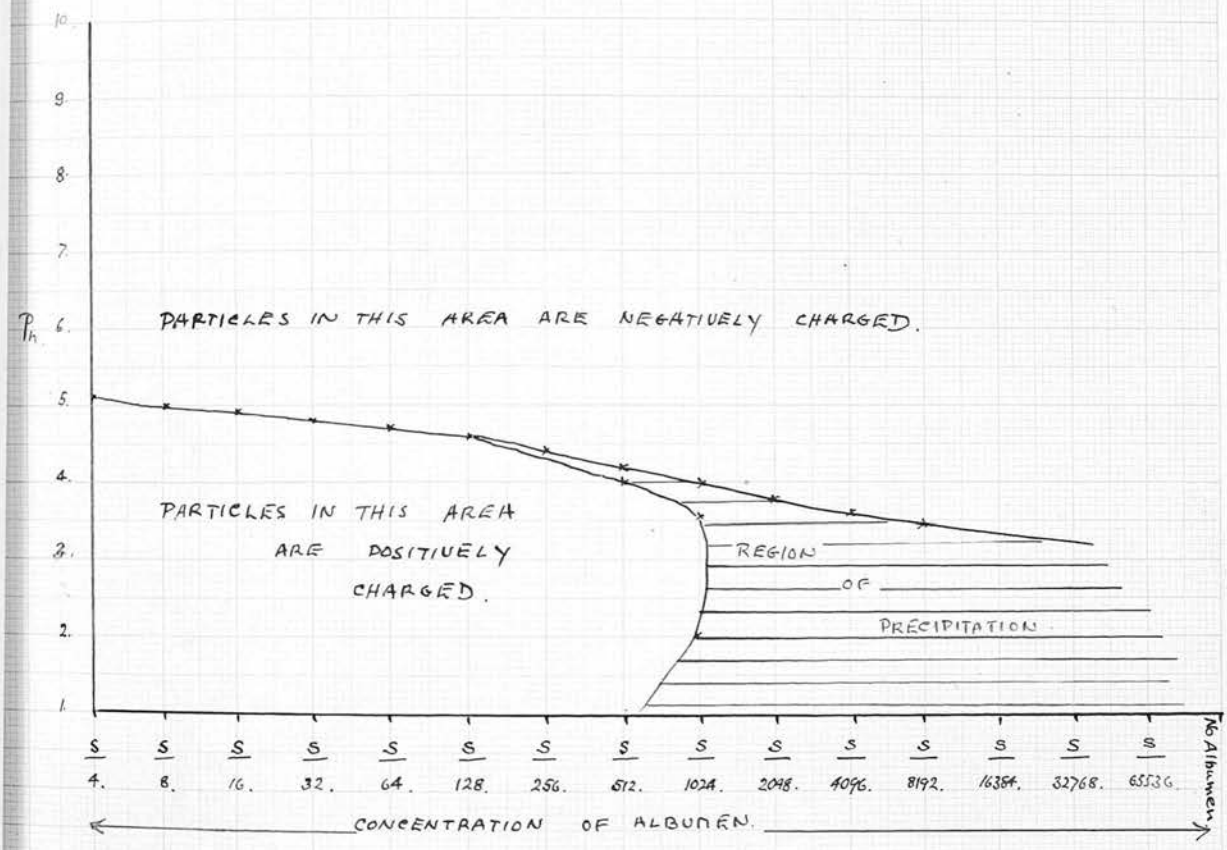

EUGLOBULIN OBTAINED FROM SYPHILITIC SERUM.
THE EUGLOBULIN USED IN THIS EXPERIMENT WAS UNHEATED.

Fig. 13.




PSEUDOGLOBULIN OBTAINED FROM SYPHILITIC SERUM.
THE PSEUDO GLOBULIN USED IN THIS EXPERIMENT WAS UNHEATED.

Fig. 14.




ALBUMEN OBTAINED FROM SYPHILITIC SERUM.

THE ALBUMEN USED IN THIS EXPERIMENT WAS UNHEATED.

A large number of factors are clearly involved and the positions not only of the region of abnormal precipit-:ation but also of the dotted line are not constant from serum to serum. In fig.( 11 ), is shown the result of a particular experiment made from a single series of tubes using serum from a case of syphilis.

When figures ( 13 and 14 ) are compared with figures ( 7 and 8 ) it is seen that there is very close agreement and that therefore albumen and pseudoglobulin are apparently not altered as the result of syphilitic infection,at least in their power to cause precipitation of colloidal gum benzoin. On the other hand fig.( 12 ) differs from fig.( 6 ) in that the region of precipit-:ation at high concentrations of protein extends to a $P_h$ 8.5 in the case of syphilitic euglobulin,whereas in the case of normal euglobulin it does not extend beyond a $P_h$ 7.4. It is therefore in the euglobulin fraction that the alteration occurs which is respons-:ible for the zone of abnormal precipitation in syphilitic serum

It is of some interest to ascertain whether the fraction which appears to be responsible for the abnormal region of precipitation, that is to say the euglobulin fraction, gives a positive result when submitted to the Wassermann and Sigma reactions and whether the other fractions,pseudoglobulin and albumen fail to do so. The fractions were therefore submitted to

Table 4.

| CONCENTRATION OF SALINE. | CONCENTRATION OF SERUM | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/2048 | 1/4096 | 1/8192 | 1/16384 | 1/32768 | $H_2O$ |
| 12% | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 4 | 4 | 4 | 4 | 4 | 4 |
| 6% | 4 | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 3% | 4 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 1·5% | 4 | 4 | 0 | 2 | 2 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 0·75% | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 0·37% | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 |
| 0·18% | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 |
| 0·09% | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 2 | 4 | 4 | 4 | 4 | 0 |
| $H_2O$ | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 0 | 0 |

SYPHILITIC SERUM

UNHEATED.

to these tests and the results are given in the following table. 5

Table. 5

| No | Euglobulin | | Pseudoglobulin | | Albumen | |
|---|---|---|---|---|---|---|
| | WaR. | Sigma | WaR. | Sigma | WaR. | Sigma |
| 1. | +++ | 11units | negative | 0.0 | neg. | 0.0 |
| 2. | +++ | 8 " | " " | 0.0 | " | 0.0 |
| 3. | +++ | 34+ " | " " | 0.0 | " | 0.0 |
| 4. | +++ | 34+ " | " " | 0.0 | " | 0.0 |
| 5. | +++ | 17 " | " " | 0.0 | " | 0.0 |
| 6. | +++ | 85 " | " " | 0.0 | " | 0.0 |

Notes.
WaR = Wassermann reaction.
+++ = Six doses of complement deviated.
neg = Complete haemolysis in 2,3 and 6 doses of complement.

It may be noted that clean cut results such as these shown in the table are obtained only if the process of dialysis, by means of which the euglobulin fraction is separated, is carried on sufficiently long to remove all electrolytes. If care is not taken to ensure that all salt is removed the separation of the euglobulin is not complete and a positive result in the Wassermann reaction may be given by the pseudoglobulin fraction.

In view of the fact that the influence of various concentrations of sodium chloride on the precipitation of gum benzoin by normal serum has already been examined, ~~Sig. (—)~~, Table 2. it was considered of interest to carry out a similar experiment using a typical syphilitic serum in place of normal serum. The result is summarised in table 4. It will be seen that no particular difference is brought to light by this method of experimentation.

4. The effect of prelimnary heating upon blood serum or related fluids.

It has already been mentioned in section 2 that the degree of precipitation which is observed in the first few tubes of a series of dilutions in distilled water of normal serum to which gum benzoin has been added in the usual way is decreased if the serum is heated for 30 minutes at 55°C before the test is carried out. It was considered desirable to investigate this point in greater detail since the previous heating of the serum has clearly an important influence on the results obtained in precipitation experiments such as form the main subject of this thesis. It will be recalled that in carrying out the Wassermann reaction sera are heat-:ed for 30 minutes at 55°C. Although the primary object of this heating is to inactivate the complement present in the serum itself, it has the further important effect of eliminating a certain number of positive (or anti-:complementary) results which may be given by normal sera if they are not thus treated. Similarily sera are heated for 90 minutes at 55°C before thay are submitted to the Sigma reaction. In this case the question of inactivating the complement does not arise, but if the sera are not thus heated, misleading, positive results may be obtained.

The results of tests carried out with one normal and one syphilitic sera are set forth in the following table. Each row corresponds to a series of dilutions in distilled water, the serum being previously heated for 0 minutes, 30 minutes, and over 90 minutes. It is seen that the chief alteration effected by heating occurs in the first four tubes.

Table 6 .

NEGATIVE SERUM. (Wassermann reaction negative Sigma reaction 0.0 units )

| Heated for:- | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | C. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 minutes | 2 | 0 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 2 | 0 |
| 30 " " | 0 | 0 | 0 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 4 | 3 | 0 |
| 90 " " | 0 | 0 | 0 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 0 |

POSITIVE SERUM.(Wassermann positive Sigma 68.0 units.)

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 minutes | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 4 | 3 | 2 | 1 | 0 |
| 30 " " | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 4 | 3 | 2 | 1 | 0 |
| 90 " " | 0 | 0 | 0 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 1 | 0 |

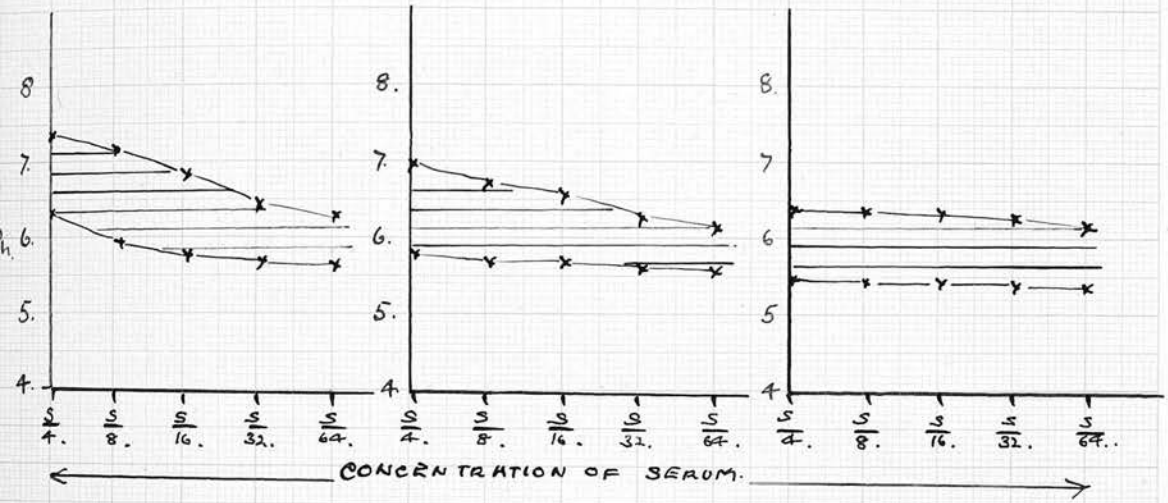
Concentration of serum in tube 1 = $\frac{1}{2}$
" " " " " " " " " 2 = $\frac{1}{4}$
" " " " " " " " " 3 = $\frac{1}{8}$
C = Control, contains distilled water, no serum.

In order ~~to~~ more closely to investigate the matter a more extended series of observations has been carried out using only the first four dilutions. The results are represented in table 7 .

These figures confirm the result previously found that less precipitation occurs if the serum has been previously heated. It appears that even in the case of a positive serum , complete absence of precipitation may be found in the first four tubes if the serum has been previously heated for 120 minutes.

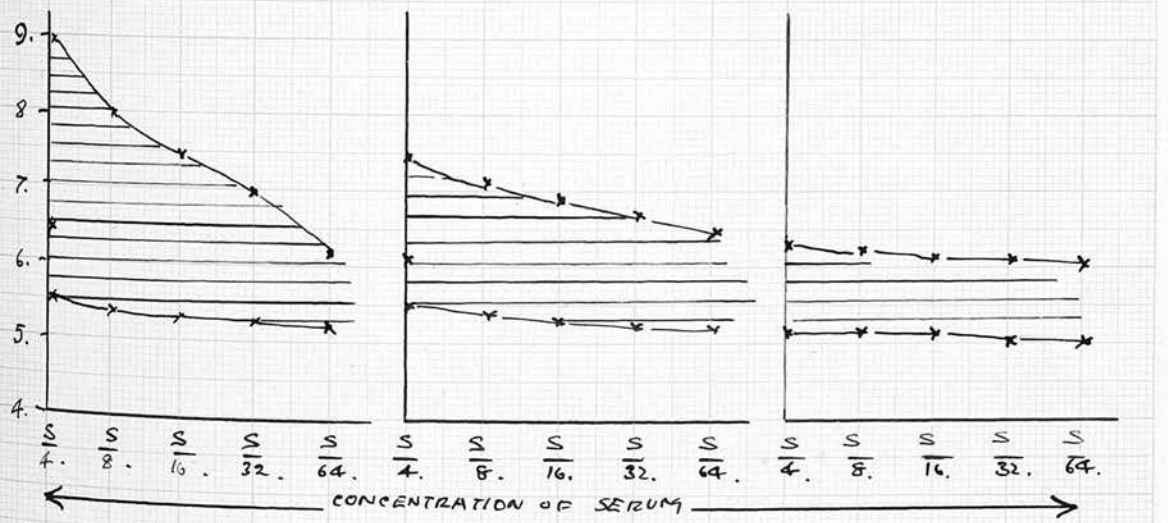
UNIV. EDIN.

Fig. 15.




NORMAL SERUM.

NO HEATING. HEATING 15 MINS AT 55°C. HEATING 30 MINS AT 55°C.




SYPHILITIC SERUM.

NO HEATING. HEATING 75 min AT 55°C. HEATING 120 MINS at 55°C.

ASEPTIC FLUID USED IN THESE EXPERIMENTS.

THE ABOVE SHOW THE CHANGES IN THE FIRST ZONE OF PRECIPITATION UPON HEATING.

REGION OF PRECIPITATION ≡

Table 7.

| Time heated at 55°C. | Normal Serum | Syphilitic serum |
|---|---|---|
| 0 minutes | 2.0.4.4. | 4.4.4.4. |
| 5 " " | 0.0.4.4. | 4.4.4.4. |
| 10 " " | 0.0.2.4. | 4.4.4.4. |
| 15 " " | 0.0.0.4. | 4.4.4.4. |
| 20 " " | 0.0.0.3. | 4.4.4.4. |
| 25 " " | 0.0.0.3. | 4.4.4.4. |
| 30 " " | 0.0.0.2. | 3.4.4.4. |
| 35 " " | 0.0.0.0. | 2.3.4.4. |
| 40 " " | 0.0.0.0. | 2.2.3.4. |
| 120 " " | 0.0.0.0. | 0.0.0.0. |

The greatest contrast between negative and positive sera is seen to exist after they have been heated for 25-30 minutes. The effect of heat was also examined by the extended method previously described in which the hydrogen ion concentration as well as the concentration of the protein are varied independently. In these experiments however only the first six dilutions were prepared and because of the large quantity of material required it was found convenient to use ascitic fluid in the place of serum. Fig. (15) therefore refers to ascitic fluid. It will be seen that the result of previous heating of the ascitic fluid a change takes place in the region lying between $P_h$ 5.5 and $P_h$ 9.0. In the case of the normal ascitic fluid __ that is to say an ascitic fluid from a person not suffering from syphilis __the region of precipitation which occurs high concentrations of of the fluid extends from a $P_h$ 6.3 to Ph 7.4 but after heating for 30 minutes this region of precipitation does not above a$P_h$ 6.4. In the case of an ascitic fluid giving strongly positive results

Table 8.

| CONCENTRATION OF ACID OR ALKALI | CONCENTRATION OF SERUM. | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/2048 | 1/4096 | 1/8192 | $H_2O$. |
| N/40 HCl | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 4 | 4 | 4 |
| 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 4 | 4 |
| 160 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 |
| 320 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 |
| 640 | 3 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 4 | 2 | 1 | 0 |
| 2·80 | 0 | 1 | 4 | 4 | 4 | 0 | 0 | 4 | 4 | 3 | 0 | 0 | 0 |
| 5120 | 0 | 0 | 3 | 3 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 |
| $-H_2O$ | 0 | 0 | 0 | 3 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| 400 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| N/40 NaOH | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

THIS EXPERIMENT HAS BEEN CARRIED OUT WITH ASCITIC FLUID.

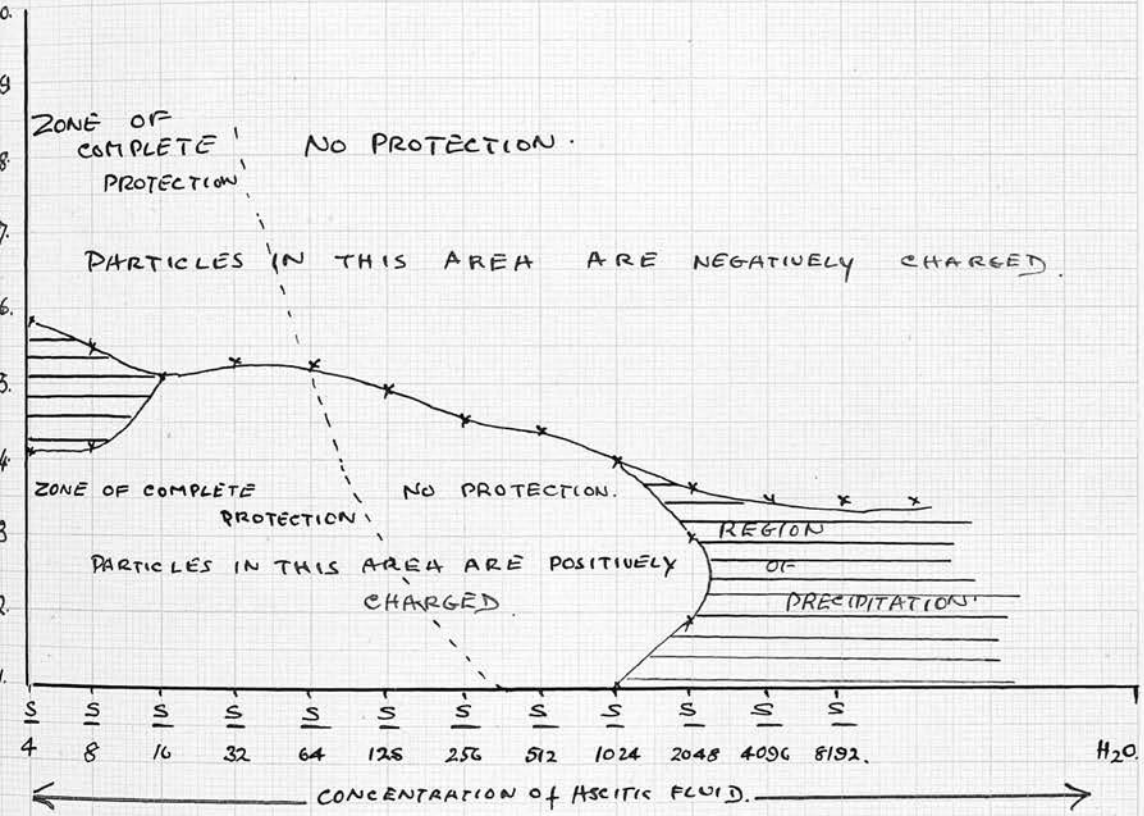
THIS FLUID WAS NEGATIVE TO THE WASSERMANN REACTION.

HEATED 18 HOURS AT 55°C.

COMPLETE PROTECTION DENOTED 0

HALF PROTECTION DENOTED 0'

Fig 16.




ASCITIC FLUID

HEATED 18 HOURS AT 55°C.

with the Wassermann and Sigma reactions the figure referring to unheated fluid shows a region of precipit-:ation extending from Ph 5.5 to Ph 9.0 and after heating for 30 minutes is practically unaltered. When however heating is continued for 120 minutes the region does not extend above Ph 6.3. It appears therefor that the region of precipitation characteristic of syphilitic fluids fails to occur after these fluids have been heated at 55°C for two hours. Table 8. and fig.(16) which gives the results of the complete examination of a normal ascitic fluid after it had been heated for 18 hours at 55°C may also be referred to here. This figure shows that the zone of normal precipitation occurring at relatively high dilutions is essentially unaltered by the previous heating. In other respects the diagram is consistent with those previously given in this section.

The theoretical explanation of the effect of previous heating of the serum on the region of precip-:itation which occurs at high concentrations presents considerable difficulty, and no definite conclusion appears possible at present. It is possible however that the phenomenon is related to an incipient coag-:ulation of the serum, and if this were so it might be associated with the increase in size of certain of the protein micellæ . The surface of the micellæ would be increased and their precipitating action

diminished. In this way the gradual disappearance of the regions of precipitation as the period of heating is prolonged would be accounted for. The whole matter appears to demand further investigation in order that any definite conclusion can be arrived at.

These experiments with ascitic fluid are of interest in that they demonstrate that in the case of fluids giving a positive result in the Wassermann and Sigma reactions a region of precipitation is obtained very closely similar to that found in the cases of blood serum and of cerbro spinal fluids giving a positive Wassermann and Sigma reaction.

The fact that body fluids the protein content of which range from that of cerebro spinal fluid to that of ascitic fluid and blood serum would seem to indicate that its appearance is dependant not on the ordinary albumen and globulin but on some other fact apart from these proteins.

5. The examination of normal serum and normal serum fractions to which clupeine sulphate has been added.

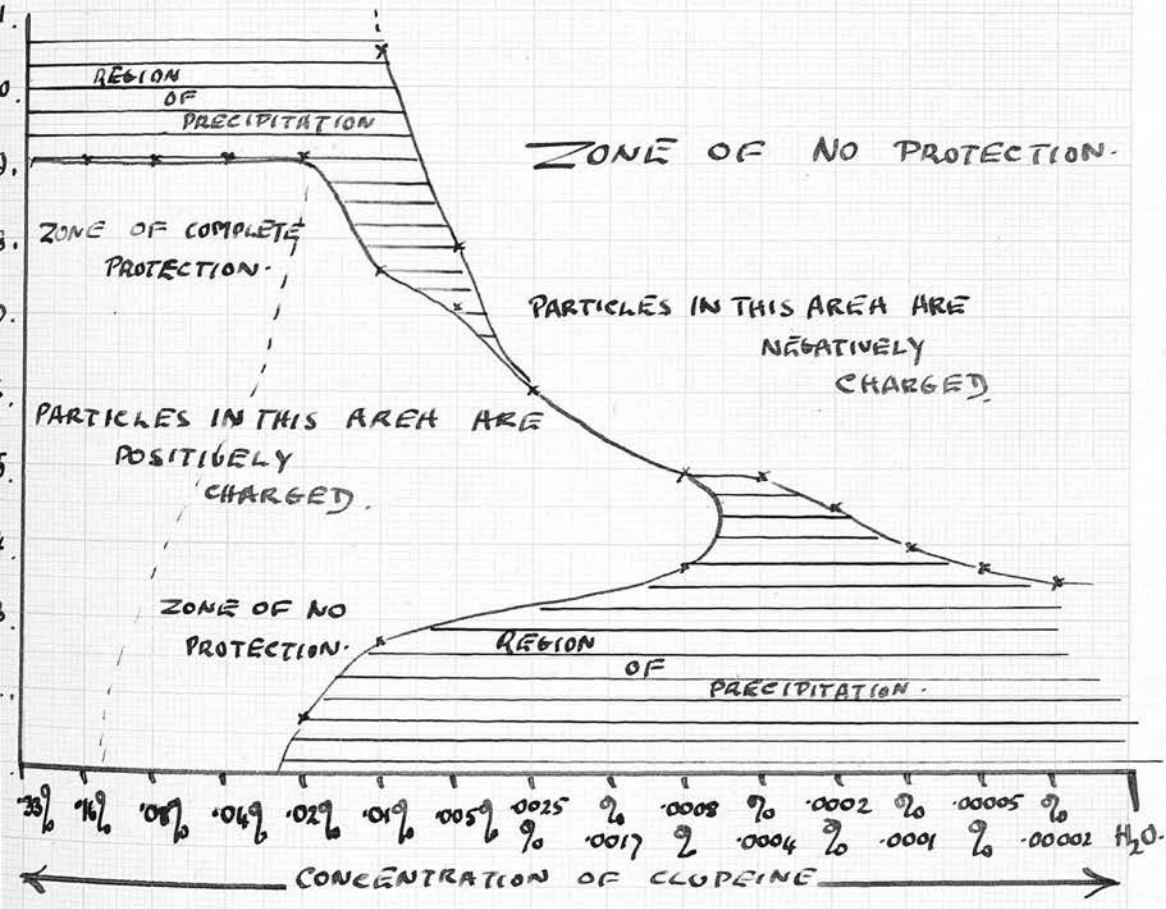
It has already been stated in the introduction to this thesis that the suggestion was made by Wright and Kermack that the region of precipitation lying between $P_h 5$ and Ph 8 which appears when cerebro spinal fluid from a case of general paralysis of the insane is examined, might be occasioned by the presence in it of a protein or other compound the isoelectric point of which lies well above Ph 6. It has been shown in the previous section that a region closely analogous to that which is observed in the case of cerebro spinal fluid from a case of general paralysis of the insane is found also when serum or ascitic fluid giving a positive Wassermann reaction is examined in a similar way. The smae considerations then lead to the suggestion that in the cases of serum or ascitic fluid also some substance with a relatively high isoelectric point may be the cause of the characteristic precipitation in this region which may conveniently be called the syphilitic region.

In order to investigate this hypothesis experimentally it was decided to prepare a protein of high isoelectric point and to find out whether negative serum showed precipitation in the syphilitic region when small quantities of this protein had been

to it. Of the various types of proteins the class of protamines is characterised by the fact that it's mem-:bers possess a high isoelectric point usually in the neighbourhood of $P_h$ 12. Protamines are relatively simple proteins and certain of them are readily acces-:sable. Amongst these one of the best known is clupeine which was first prepared by Kossel (6), from the milt of the herring. A detailed account of the preparation is given in the appendix to this thesis. Clupeine like other proteins is built up of amino acids but in this particular case the most important amino acid is arginine ( $\delta$ guanidine $\alpha$ amino valeric acid ). In consequence of it's large content of arginine, clupeine is relatively basic in it's behaviour, a fact which is expressed in the value of it's isoelectric point, $P_h$ 12. It follows that when the $P_h$ value of a solution containing clupeine is less than 12, the protamine will exist in the form of cations and will possess a positive charge. It should thus be able to effect precipitation of negatively charged colloidal suspensions at $P_h$ values much higher than those at which serum globulin or serum albumen can do so.

It is therefore of interest first of all to examine a solution of clupeine sulphate by the general methods which have been used in the previous sections.

Fig. 16a




Precipitating effect of Clopeine Sulphate upon Gum Benzoin Sol.

# Table 9.

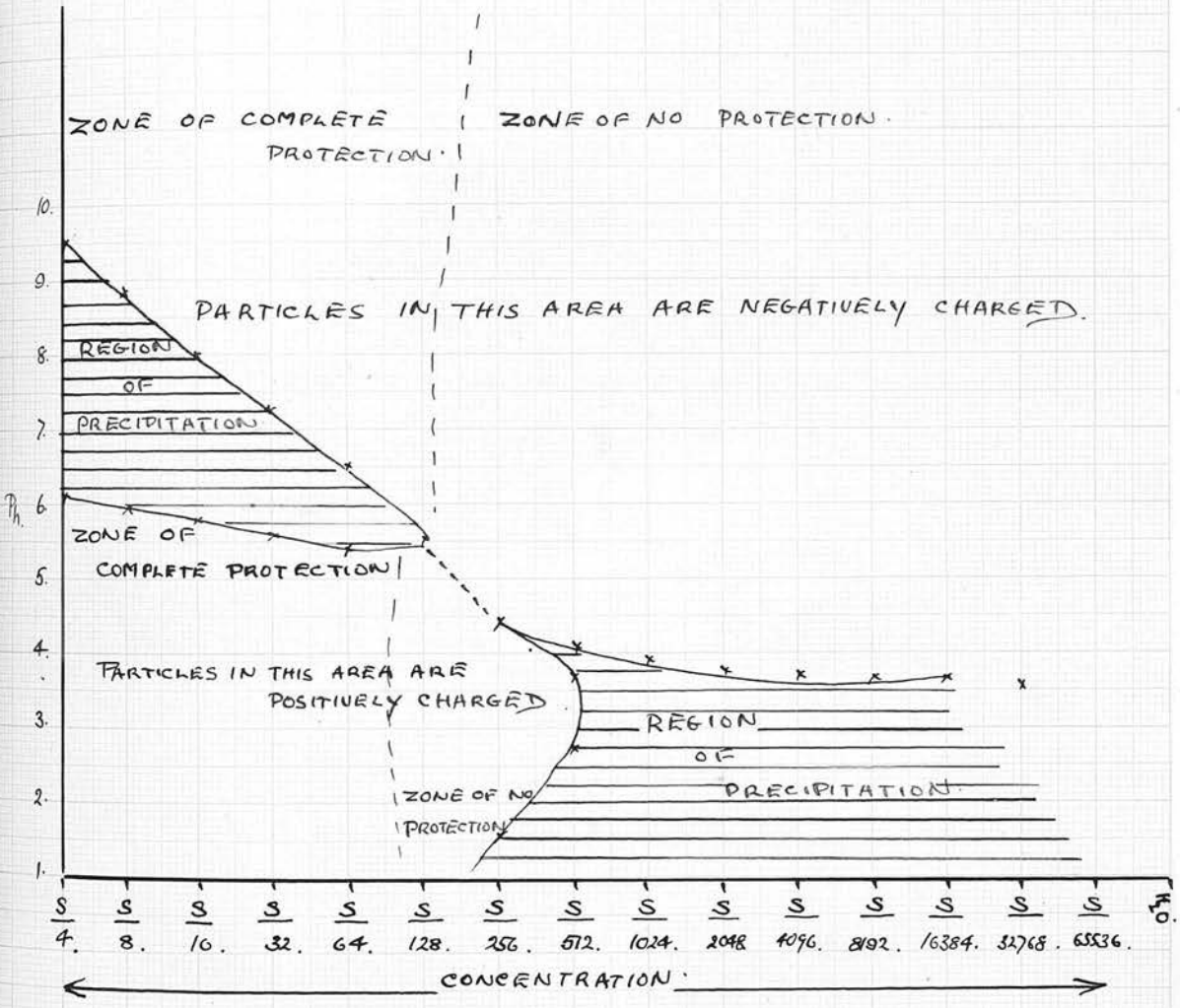
| CONCENTRATION OF ACID OR ALKALI. | CONCENTRATION OF CLUPEINE SULPHATE | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0·33% | 0·16% | 0·08% | 0·04% | 0·02% | 0·01% | 0·005% | 0·0025% | 0·0017% | 0·0008% | 0·0004% | 0·0002% | 0·0001% | 0·00005% | 0·00003% | $H_2O$. |
| N/40 HCl | 0 | 0 | 2 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 400 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 4000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 0 | 0 |
| $-H_2O$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 0 |
| 1280 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 640 | 0 | 0 | 0 | 0 | 0 | 2 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 320 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 3 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 160 | 4 | 0 | 0 | 0 | 4 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 80 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| N/40 NaOH | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

CLUPEINE SULPHATE

AREA OF PRECIPITATION BEFORE ADDITION OF BENZOIN SOL ≡

sections. The result is summarised in table 9 and fig. 16a. In the latter the ordinates represent as usual the $P_h$ values, and the abcissae the concentrations of clupeine sulphate in the final mixtures after the addition of colloidal gum benzoin. It will be observed that two regions exist in which no precipitation takes place and that in one of these the particles bear a negative charge, whilst in the other they bear a pos-:itive charge. These two regions are separated by a region of precipitation in many ways closely analogous to the regions of precipitation which are found in many of the figures in the previous sections. It differs from them however in one important respect. Instead of stopping at about $P_h$ 5 it extends till it reaches $P_h$ 9 and the region of positive charge also extends to $P_h$ 9. This difference is of course to be expected because of the high isoelectric point of the clupeine. It might in fact have been anticipated that the region of positive charge would extend up to a $P_h$ 12 but certain complicating factors produce an effect which obscure the phenomenon at a higher $P_h$. In order to increase the $P_h$ considerable quantities of sodium hydroxide must be added. This alkali reacts with clupeine sulphate so as to liberate clupeine and form sodium sulphate and the precipitation observed at a $P_h$ 9 - 12 is at least partly to be accounted for

Fig. 17.




0·5 c.c NORMAL SERUM + 0·5 cc. 1·35% CLUPEINE SULPHATE = $\frac{S}{1}$

Precipitating effect of the above mixture upon Gum Benzoin Sol.

Table 10

| CONCENTRATION OF ACID OR ALKALI | ← CONCENTRATION OF NORMAL SERUM and CLUPEINE SULPHATE → | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/2048 | 1/4096 | 1/8192 | 1/16384 | 1/32768 | 1/65536 | $H_2O$ |
| N/40 HCl | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 |
| 160 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 |
| 320 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 4 | 4 | 4 | 4 |
| 640 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 0 | 0 | 0 |
| 1280 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 4 | 4 | 0 | 0 | 0 |
| 2580 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 3 | 0 | 0 | 0 |
| 5120 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 0 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 0 |
| − $H_2O$ | 4 | 4 | 4 | 4 | 4 | 4 | 2 | 3 | 3 | 4 | 4 | 0 | 0 | 0 | 0 | 0 |
| 400 | 4 | 4 | 4 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| N/40 NaOH | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

NORMAL SERUM to which CLUPEINE SULPHATE has been added.

9·5 cc. normal serum + 0·5 cc. 1·35% Clupeine Sulphate. = S/1

COMPLETE PROTECTION DENOTED 0

HALF PROTECTION DENOTED 0

by the presence in the tubes of sodium sulphate which itself tends to precipitate colloidal gum benzoin.

In order to investigate the effect upon normal serum of the addition of clupeine sulphate 0.5c.c of a solution containing 1.35% clupeine sulphate was added to 9.5c.c. of normal serum and the resulting mixture was examined in the usual way. The results are summarised in table **10** , and fig.17 . It will at once be seen that precipitation occurs in what we have called the syphilitic region and that there is a very close similarity between fig.17 and fig.10 which refers to serum from a case of syphilis. It therefore appears that the presence in normal serum of a small quantity (0.07%) of a protamine of high isoelectric point such as clupeine is sufficient to cause it to give a characteristic precipitation in the syphilitic region when examined by the method used in this thesis.

In section 3 it was shown that when the various protein fractions of serum, euglobulin, pseudoglobulin and albumen are examined, precipitation in the syph-:ilitic region occurs only in the case of euglobulin from syphilitic sera, and not in the case of the other two fractions, whether of syphilitic origin or not. It was therefore of interest to ascertain the results of the addition of clupeine sulphate to the three protein fractions of normal serum.

To 9.5 c.c. of euglobulin solution prepared as described above 0.5c.c. of 1.35% clupeine sulphate was added and the precipitating action of this mixture upon gum benzoin sol examined. The result is summarised in table II and fig. 18. This figure may be compared with figs. 12 and 6 which refer to the euglobulin fractions of syphilitic and normal serum respectively.

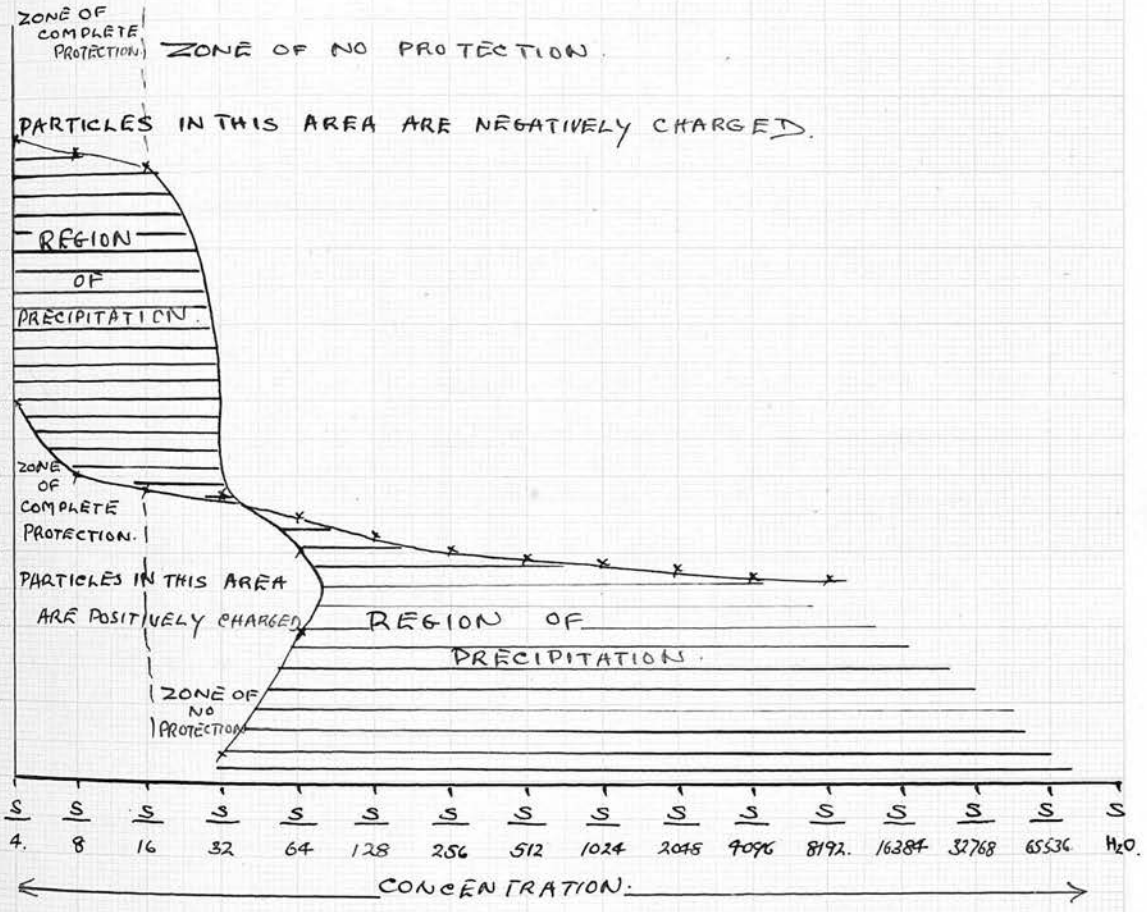
It will be noticed that in fig 18 as in fig.12 precipitation occurs in the syphilitic region.

On the other hand pseudoglobulin and albumen containing clupeine sulphate do not effect precipitation in the syphilitic region. Albumen in fact appears to inhibit the precipitating action of clupeine sulphate a fact which is of special interest in view of certain results described in the succeeding section.

It will thus be seen that when small amounts of clupeine sulphate are added to the protein fractions of normal serum, precipitation phenomena are observed very closely resembling those obtained by the use of corresponding fractions of syphilitic serum.

It is clearly of interest also to investigate the result of adding clupeine sulphate to a normal cerbro spinal fluid. A systematic examination of the resulting phenomena has not been made, but the following experiment shows that a normal cerbro spinal fluid containing clupeine sulphate gives a result very like

Fig. 18.




9·5 cc. Normal Euglobulin + 0·5 cc. 1·35% CLUPEINE SULPHATE = $\frac{S}{1}$

Precipitatory effect of the above mixture on Gun Benzoin sol.

Table II.

| CONCENTRATION OF ACID OR ALKALI. | ← CONCENTRATION OF NORMAL EUGLOBULIN and CLUPEINE SULPHATE → | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/2048 | 1/4096 | 1/8192 | 1/16384 | 1/32768 | $H_2O$ |
| N/40 HCl | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 80. | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 160 | 0 | 0 | 0 | 0 | 0 | 2 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 320 | 0 | 0 | 0 | 0 | 0 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 640 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 |
| 1280 | 4 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $-H_2O$ | 4 | 3 | 0 | 3 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4000 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 400 | 4 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| N/40 NaOH | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

NORMAL EUGLOBULIN to which CLUPEINE SULPHATE has been added.

9.5 cc. normal Euglobulin + 0.5 cc. 1.35% Clupeine Sulphate. = $\frac{S}{1}$.

COMPLETE PROTECTION DENOTED 0

HALF PROTECTION DENOTED $\frac{1}{2}$ 0

that obtained from a cerebro spinal fluid from a case of general paralysis of the insane.

Traces of clupeine sulphate, (1 drop of a 1.35% sol-:ution), were added to 2c.c. of normal cerbro spinal fluid and a series of dilutions of this mixture in distilled water were prepared. A similar series of dilutions were also prepared of normal fluid, contain-:ing no clupeine sulphate, and one from a case of gen-:eral paralysis of the insane. A series of dilutions of clupeine sulphate , (1.35%), in distilled water was also made. The contents of each tube were then diluted with an equal volume of colloidal gum benzoin. Readings made after 24 hours are shown in table 12.

Table 12.

| Material examined. | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/2048 | 1/4096 | 1/8192. | $H_2O$. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Normal C.S.F. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C.S.F. from G.P.I. | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 2 | 0 | 0 | 0 | 0 | 0 |
| Normal C.S.F. + clupeine sulphate | 4 | 4 | 4 | 4 | 4 | 4 | 1 | 0 | 3 | 4 | 0 | 0 | 0 | 0 |

| Clupeine sulphate alone.. | 0·67% | 0·33% | 0·16% | 0·08% | 0·04% | 0·02% | 0·01% | 0·005% | 0·0025% | 0·0012% | 0·0006% | 0·0003% | 0·0001% | $H_2O$. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 0 | 0 | 0 |

From the above it appears that in the cases of normal cerbro spinal fluids and with clupeine sulphate alone no precipitation occurs in the first nine dilutions but that the addition of even a faint trace of clupeine sulphate to a normal fluid causes precipitation to occur in the first five tubes and also in tubes 8 and 9, a degree of precipitation

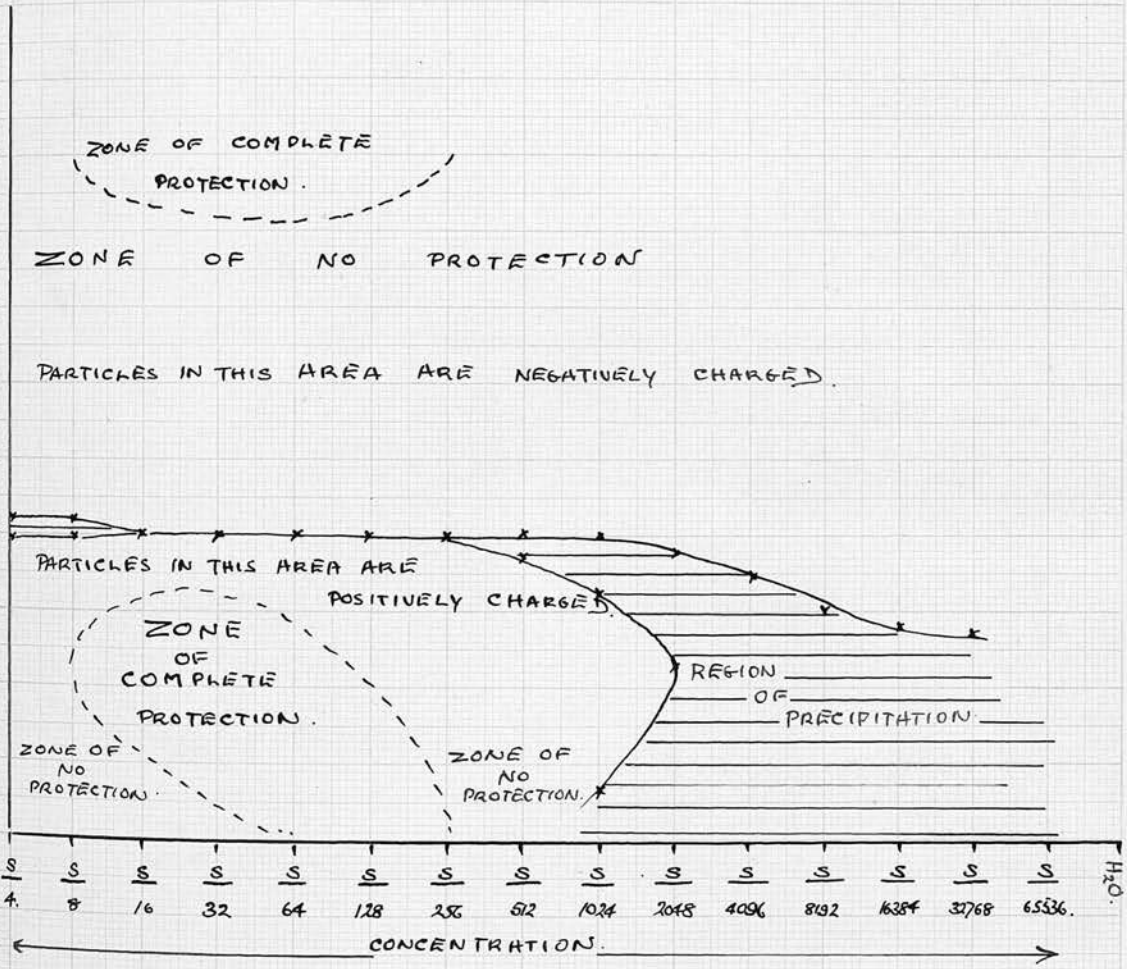
Table 13.

| CONCENTRATION OF ACID OR ALKALI | CONCENTRATION OF SERUM-TANNIN MIXTURE | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/2048 | 1/4096 | 1/8192 | 1/16384 | 1/32768 | $H_2O$ |
| N/40 HCl | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 |
| 160 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 |
| 320 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 |
| 640 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 4 |
| 2560 | 0 | 0 | 3 | 4 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 2 | 0 |
| 5120 | 0 | 0 | 0 | 3 | 4 | 4 | 3 | 0 | 4 | 3 | 1 | 0 | 0 | 0 | 0 |
| $H_2O$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 0 |
| 400 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| N/40 NaOH | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

NORMAL SERUM to which TANNIN has been added.

3 c.c. Normal Serum + 1·5 c.c. 2% Tannin = $\frac{S}{1}$.

Fig. 19.




3c.c. NORMAL SERUM + 1.5c.c 2% TANNIN = $\frac{S}{1.}$

The Precipitating effect of this mixture upon Gum Benzoin Sol.

which closely resembles that which is obtained with a fluid from a case of general paralysis of the insane. This experiment therefore indicates that a systematic investigation of this phenomenon is likely to prove of interest.

It is natural to enquire whether compounds of the type of clupeine sulphate are the only ones which when added to normal serum cause it to give results charac-:teristic of syphilitic serum. It is clearly impossible in a limited space of time to examine throughly the effects of a large number of different substances and the only one which will be treated here is tannin, which has been chosen for reasons which will be stated in the following section.

Tannin itself acts on colloidal gum benzoin like a simple acid and effects precipitation when present in sufficiently high concentration to reduce the $P_h$ to 3 or any lower value. The results of an experiment carried out in the usual manner upon normal serum (3c.c) containing tannin, (1.5c.c. of a 2 per cent solution), are shown in table 13 and fig. 19. It is at once clear that no precipitation occurs in the syphilitic region, and so at least under the conditions of the experiment tannin does not cause normal serum to simulate syphilitic serum.

6 ). Application of the Wassermann reaction to normal serum and normal serum fractions containing Clupeine.

It has been shown in the previous section that normal serum and normal serum fractions containing a small quantity of clupeine sulphate closely resemble syphilitic serum and the corresponding protein fractions from syphilitic serum in their precipitating action on colloidal gum ben-:zoin. It was thought that further evidence might be obtain-:ed as to the significance of the previous result by ascer-:taining to what extent mixtures so prepared were able to cause fixation of complement when examined by the Wassermann reaction. Several references were found in the literature dealing with the effect of the addition of various subs-:tances to normal serum in altering their behaviour to the Wassermann reaction. For instance, Mahlo ( 7 ) has stated that the addition of glycine to normal serum causes it to give a positive reading. We have been unable to confirm this finding. Stern ( 8 ) has claimed that the addition of Tannin to normal serum causes it to give a positive reading. We have found that normal serum con-:taining 1% tannin does in fact deviate at least 25 doses of complement, but the same number of doses of complement are fixed when no antigen is added. In other words blood serum containing tannin is very strongly anticomplementary, and so it appears that no significance can be attached to the result. This experiment however suggests the importance of ascertaining to what extent

any particular serum is anticomplementary when it is altered by the addition of a foreign substance.

It may be added here that it was because of the claim of Stern , ( 8 ), with regard to tannin that the precipitation experiments with this substance, which have been described in the previous section were carried out. It will be recalled that the evidence of these experiments also indicates that the alterations which occur in blood serum as a result of syphilitic infection are not in any way analogous to those produced by the addition of tannin.

Two methods of carrying out the Wassermann reaction have been used. The first , method A is that employed in the Royal College of Physicians laboratory. It is essentially that recommended by the Medical Research Council, (No.4), using a Bordet antigen in place of that of MacIntosh and Fildes. The second method, B is that employed by Professor Mackie. The full details of these two methods are fully described in the appendix. In the following experiments the method employed is indicated in each instance, but in the case of method B, the actual quantities employed will be stated in each case.

The following abreviations are used throughout this section :-

| | |
|---|---|
| o = no haemolysis | m = marked haemolysis |
| t = trace of haemolysis | ac = almost complete " |
| d = distinct haemolysis | c = complete haemolysis |

Preliminary to the examination of normal serum containing clupeine sulphate it was desireable to investigate the reactions given by clupeine sulphate alone. To this end the following experiment was carried out. A 1 % solution of clupeine sulphate in 0.85% sodium chloride solution was prepared care being taken to adjust the reaction to $P_h$ 8, by addition of sodium hydroxide solution. To each tube was added 0.2 c.c. of this solution, 0.2c.c. of a solution containing the appropriate amount of complement, and finally 0.2 c.c. of antigen or of normal saline as the case may be. These mixtures were heated at 37°C for 30 minutes, sensitised cells were then added and the reaction carried out as in method A. the results are shown in the following table. (14)

Table 14.

| | Doses of Complement | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 6 | 10 | 14 | 18 | 22 | 26 | 30 | 40 |
| ANTIGEN | - | m | d | o | o | t | d | d | d | ac |
| NO ANTIGEN | c | c | c | ac | ac | c | t | t | t | t |

From the above table it will be evident that clupeine sulphate in the absence of complement has a haemolytic action. When more than 6 doses of complement are present little or no haemolysis occurs unless 22 or more doses have been added.

Table 15.

| | ANTIGEN | | | | | NO ANTIGEN. | | | |
|---|---|---|---|---|---|---|---|---|---|
| | DOSES OF COMPLEMENT | | | | | | | | |
| MATERIAL TO BE EXAMINED | 2. | 4. | 6. | 9. | 14. | 2. | 4. | 6. | 9. |
| 2 cc. Saline + 0.3 cc. Clupeine Sulphate | f | f | m | vm | c | o | c | c | c |
| " " " + 0.5 " " " " | o | f | d | ac | c | d | m | ac | c |
| " " " + 0.6 " " " " | o | o | f | m | c | o | o | o | d |
| " " " + 0.7 " " " " | o | f | d | ac | c | o | f | f | ac |
| " " " + 0.9 " " " " | o | o | f | ac | c | o | f | ac | c |

Fixation of complement in presence and absence of antigen by varying concentrations of Clupeine Sulphate.

Method B was used.

Table 16.

| | ANTIGEN | | | | | NO ANTIGEN | | | |
|---|---|---|---|---|---|---|---|---|---|
| | DOSES OF COMPLEMENT | | | | | | | | |
| | 2 | 4 | 6 | 9 | 14 | 2 | 4 | 6 | 9 |
| 30 mins Heating at 55°C | o | f | ac | c | c | d | m | c | c |
| " " " 58°C | o | f | ac | c | c | d | m | c | c |
| " " " 62°C | o | f | d | ac | c | f | d | ac | c |
| Solution Boiled for 2 mins | o | f | ac | c | c | d | m | c | c |
| Solution unheated | o | f | ac | c | c | d | m | c | c |

Effect of Heat upon the fixation of complement by Clupeine Sulphate in the presence and absence of antigen.

Method B was used.

It would appear that the proteins present in the serum added as complement inhibits the haemolytic action of the clupeine sulphate. At the same time the clupeine sulphate inactivates the complement, unless the latter is present in large quantity. Table 15. shows the fixation of complement given by different concentrations of clupeine sulphate in saline. This experiment was carried out by method B. The solutions of clupeine sulphate were prepared by adding a suitable quantity of 1 % solution to 2 c.c. of 0.85 % sodium chloride solution. These mixtures were diluted with saline to 8 c.c. and 0.2 c.c. of the resulting solution was added to the respective tubes along with the necessary quantities of antigen and complement.

Table 16 summarises the results of experiments carried out using solutions of clupeine sulphate which have been heated at various temperatures, method B being used in this experiment. In this case 0.05c.c. of 0.1% solution of clupeine sulphate was added to the tubes along with the necessary amounts of antigen and complement . It will be seen that no alteration is produced even by boiling the solution. This is of course to be anticipitated since clupeine sulphate is a stable substance and solutions of it are unaltered by boiling.

The results of the examination of normal serum to which clupeine sulphate has been added in different concentrations is shown in table , 17, (carried out by method A) and table 18, (carried out by method B.) The solutions used in these experiments were prepared by the addition of a suitable quantity of 1% clupeine sulphate solution to 2 c.c. of normal serum. In the case of the second experiment carried out according to method B, these mixtures were diluted with saline to 8 c.c., and 0.2c.c of the resulting dilution was added to the respective tubes along with the necessary quantities of antigen and complement. In the case of table . the mixtures of clupeine sulphate solution and serum were diluted with sodium chloride solution to 5 times their original volume and 0.2c.c. of these diluted solutions were used for the experiment. For the sake of comparison, in table 18, the corresponding results derived from these experiments, (in which serum was absent ), are included.

It appears from these results that normal serum to which clupeine sulphate has been added in suitable concentration is able to effect the fixation of 6 doses of complement in the Wassermann reaction under conditions such that no fixation occurs in the absence of antigen. It may be stated here that these experiments have been repeated several times, some of them under the supervision of Professor Mackie.

TABLE 17.

| Material to be examined. | Heated at 55°C for the following number of minutes. | ANTIGEN | | | | | NO ANTIGEN | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DOSES OF COMPLEMENT. | | | | | | | | | |
| | | 2 | 3. | 6. | 8. | 10. | 2 | 3. | 6. | 8. | 10. |
| Normal serum alone | 30 | c | c | c | c | c | c | c | c | c | c |
| 1 cc. " " + 0.5 c.c. Cholesterin Sulphat. | 30 | o | o | o | ac | c | o | o | c | c | c |
| " " " " " " " | 40 | o | o | ac | c | c | o | o | c | c | c |
| " " " " " " " | 50 | o | d | ac | c | c | o | o | c | c | c |
| " " + 0.7 cc " " | 30 | o | o | o | d | m | o | o | d | c | c |
| " " " " " " " | 40 | o | o | o | c | c | o | o | ac | c | c |
| " " " " " " " | 50 | o | o | ac | c | c | o | o | c | c | c |
| " " + 0.9 c.c. " " | 30 | o | o | o | ac | c | o | o | m | c | c |
| " " " " " " " | 40 | o | o | f | m | c | o | o | ac | c | c |
| " " " " " " " | 50 | o | o | m | ac | c | o | o | ac | c | c |

The influence of length of time of heating (at 55°C) upon the fixation of complement by Cholesterin Sulphate in the presence & absence of antigen

Method A was used.

# Table 18

| Material under investigation. | Heated for 30 mins at —°C. | Antigen — Doses of complement 2. | 4. | 6. | 9. | 14. | 19. | No Antigen — Doses of complement 2. | 4. | 6. | 9. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Normal serum alone. | 55° | c | c | c | c | c | c | c | c | c | c |
| 2cc. Normal serum + 0.3 cc. Chapein Sulphate | 55° | t | c | c | c | c | c | m | c | c | c |
| " " " " " " " " | 58° | m | c | c | c | c | c | c | c | c | c |
| " " " " " " " " | 62° | m | c | c | c | c | c | c | c | c | c |
| 2cc. 0.85% Sodium Chloride + " " " | 55° | t | t | m | vm | c | c | o | c | c | c. |
| 2cc. Normal serum + 0.5 c.c " " " | 55° | o | o | d | c | c | c | c | c | c | c |
| " " " " " " " " | 58° | o | d | ac | c | c | c | c | c | c | c |
| " " " " " " " " | 62° | d | c | c | c | c | c | c | c | c | c |
| 2cc. 0.85% Sodium Chloride + " " " | 55° | t | ac | ac | c | c | c | d | m | ac | c. |
| 2cc. Normal serum + 0.6 cc. " " " | 55° | o | d | ac | c | c | c | c | c | c | c |
| " " " " " " " " | 58° | ac | c | c | c | c | c | c | c | c | c |
| " " " " " " " " | 62° | c | c | c | c | c | c | c | c | c | c |
| 2cc. 0.85% Sodium chloride + " " " | 55° | o | o | t | t | m | ac | o | o | o | d |
| 2cc. Normal serum + 0.7 cc. " " " | 55° | t | d | ac | c | c | c | c | c | c | c |
| " " " " " " " " | 58° | o | m | c | c | c | c | c | c | c | c |
| " " " " " " " " | 62° | c | c | c | c | c | c | c | c | c | c |
| 2cc. 0.85% Sodium Chloride + " " " | 55° | o | t | ac | c | c | c | o | t | ac | c |
| 2cc. Normal serum + 0.9 cc " " " | 55° | d | ac | c | c | c | c | c | c | c | c |
| " " " " " " " " | 58° | d | c | c | c | c | c | c | c | c | c |
| " " " " " " " " | 62° | c | c | c | c | c | c | c | c | c | c |
| 2cc. 0.85% Sodium chloride + " " " | 55° | o | o | t | ac | c | c | o | t | ac | c |

The Influence of Temperature upon the degree of fixation of complement by Chapein Sulphate

Method B. was used.

In order that 6 doses of complement should be fixed it is necessary that the proper concentration of clupeine sulphate should be present and that the conditions should be otherwise suitable. Not infrequently only four doses have been fixed but over a considerable range of concentrations of clupeine it appears that a distinctly positive result is obtained. If too much clupeine is present the serum gives an anticomplementary reaction and fixation occurs even in the absence of anti-:gen.

It is well known that serum which gives a positive result in the Wassermann reaction tends to become negative if it is heated for 30 minutes at a temperature higher than 55°C or if it is heated for a long period at 55°C. This effect is particularly marked in the case of weakly positive sera. It is clear that the value of the results given above by serum containing clupeine sulphate, would be enhanced if it were shown that such mixtures likewise reacted negatively in the Wassermann reaction after similar heating. Included in the above tables are the results of experiments which have been made on this point. It will be seen that a mixture of normal serum and clupeine sulphate solution, which gave a positive result after being heated for 30 minuted at 55°C. becomes markedly weaker if heating at this temp-:erature is continued for a further period of 30 minutes

and actually negative after it has been heated at 62°C for 30 minutes. It may incidentally be noted that alth-:ough blood serum does not coagulate when heated at 62°c if it is diluted three to five times with 0.85 per cent sodium chloride solution, yet coagulation does occur if the mixture contains a sufficiently high concentration of clupeine sulphate. Referring to table 18. slight coag-:ulation had occurred in the mixture to which 0.6c.c clupeine sulphate had been added, rather more with 0.7c.c., and marked coagulation with 0.9 c.c; the coagulum however sedimented and it was possible to ascertain by inspection that complete haemolysis had occurred.

It is clearly also of very great interest to determine the effect of adding clupeine sulphate to the various protein fractions of normal serum. The details of the experiment, the results of which are shown in tables 19, 20, 21, are exactly similar to those just described, except that the fluids tested consisted of solutions of euglobulin, pseudoglobulin, and of albumen to which clupein sulphate solution had been added. All the experiments referred to in tables 19, 20, and 21, were carried out using method A. A 0.59 % solution of clupeine in 0.85% sodium chloride was employed in these experiments and mixtures of varying amounts of this solution with 10 c.c. of the solution of the serum fraction were submitted to the Wassermann

TABLE. 19.

| MATERIAL TO BE EXAMINED | ANTIGEN | | | NO ANTIGEN | |
|---|---|---|---|---|---|
| | DOSES OF COMPLEMENT. | | | | |
| | 2. | 3. | 6. | 2. | 3 |
| 10cc. Normal Euglobulin alone | c | c | c | c | c |
| " " " " +0·5cc. Mafaice | o | o | c | c | c |
| " " " " +1·0 " " | o | o | c | c | c |
| " " " " +2·0 " " | o | o | o | c | c |

The fixation of complement by normal Euglobulin to which Clupein Sulphate has been added.

TABLE 20.

| MATERIAL TO BE EXAMINED | MINS Heated AT 55°C | ANTIGEN DOSES OF COMPLEMENT. 2. | 3. | 6. | NO ANTIGEN 2. | 3. |
|---|---|---|---|---|---|---|
| 10 cc. Normal EUGLOBULIN ALONE | 0 | c | c | c | c | c |
| " " " " " " | 30 | c | c | c | c | c |
| " " " " " " | 60 | c | c | c | c | c |
| " " " + 1 cc. 0·5% Clupeine Sulphate | 0 | o | o | c | c | c |
| " " " " " " " | 30 | o | c | c | c | c |
| " " " " " " " | 60 | c | c | c | c | c |
| " " " + 2 cc. " " " | 0 | o | o | o | c | c |
| " " " " " " " | 30 | o | o | c | c | c |
| " " " " " " " | 60 | c | c | c | c | c |

The effect of heating for varying periods of time upon normal Euglobulin to which Clupein Sulphate has been added. The degree of fixation of complement is shown both in the presence & absence of antigen.

TABLE 21.

| Amount of Euglobulin Solution. | Amount of Albumen Solution | Volume of 0·5% Glycerin Saline added | ANTIGEN | | | NO ANTIGEN. | |
|---|---|---|---|---|---|---|---|
| | | | DOSES OF COMPLEMENT | | | | |
| | | | 2. | 3. | 6. | 2. | 3. |
| 10 c.c. | 0·0 c.c. | 0·5cc | o | o | c | c | c |
| 7.5c.c. | 2·5 c.c | " | o | o | c | c | c |
| 5.0c.c. | 5·0 c.c | " | o | d | c | c | c |
| 2.5c.c. | 7·5 c.c | " | o | c | c | c | c |
| 0·0c.c. | 10·0 c.c | " | c | c | c | c | c. |
| 10·0cc. | 0·0 c.c. | 1.0cc | o | o | c | c | c |
| 7·5cc. | 2·5 c.c | " | o | o | c | c | c |
| 6·0cc. | 5·0 c.c | " | o | o | c | c | c |
| 2·5cc. | 7·5 c.c. | " | m | c | c | c | c |
| 0·0cc. | 10·0 c.c. | " | c | c | c | c | c |
| 10·0 c.c. | 0·0 cc. | 2.c.c. | o | o | o | c | c |
| 7·5 c.c. | 2·5 c.c | " | o | o | c | c | c |
| 5.0 cc. | 5·0 c.c. | " | o | c | c | c | c |
| 2·5 c.c | 7·5 c.c. | " | d | c | c | c | c |
| 0·0 c.c. | 10·0 c.c. | " | c | c | c | c | c |

The effect / the addition / a const volume / Glycerin Saline to Euglobulin and albumen in varying proportions.

reaction using method A, each mixture being diluted five times with 0.85 % sodium chloride solution before use. It will be seen from the tables that in the case of euglobulin, but not in the case of albumen, fixation of complement occurs when a suitable quantity of clupeine sulphate is present under conditions such that anticomplementary effects are not observed . It may also be noted that after heating of the euglobulin and clupeine sulphate mixtures at 55°C for over 30 minutes they fail to fix complement under the conditions of the experiment. It also appears that when mixtures of euglobulin and albumen are used, only those mixtures containing a relatively large amount of euglobulin give a positive result in the Wassermann reaction, after the addition of clupeine sulphate and that with the increasing concentration of albumen progressively less complement is fixed.

In connection with this result it may be recalled that in the experiments in the precipitation of colloidal gum benzoin, described in a previous section it was found that in presence of serum albumen no precipitation occurred in the syphilitic region, (used in the sense mentioned in page 66 ), even when clupeine sulphate is present.

On the contrary serum albumen appeared to have a markedly protective effect and retarded the precipitating action of clupeine sulphate.

It is impossible at present to conclude definitely that any connection exists between this phenomenon and the inhibiting effects of serum albumen which are shown in the fixation experiments described above, but it is reasonable to suggest that the similarity of the effects is of real significance.

## Discussion.

In the following discussion we shall first of all state briefly the most important results which have been obtained in the experimental work described above; we shall next consider certain particular points which arise and we shall conclude with a general discussion of the problem dealt with in this thesis.

It has been shown that when the precipitating effects of normal blood serum upon gum benzoin sol are examined by the general method used in the present thesis, regions of precipitation, non precipitation, and protection are found and that these results are analogous to those obtained by Wright and Kermack with normal cerebro spinal fluid. The various protein fractions of normal serum euglobulin, pseudoglobulin and albumen give similar results although the boundaries of the various regions differ somewhat in detail.

If serum giving markedly positive Wassermann and Sigma reactions are examined in a similar way analogous regions of precipitation are found, but in addition precipitation is also observed in what we have called the syphilitic region, that is at high concentrations of serum and between $P_h 7$ and $P_h 9$.

When protein fractions of syphilitic serum are examin-
:ed, precipitation in the syphilitic region occurs only in the case of euglobulin, the graphs summarizing the results of pseudoglobulin and albumen being very simil-
:ar to those obtained with corresponding protein fract-
:ions from normal serum. If the serum is heated before examination the precipitation observed tends to be less marked and if heating at 55°C is sufficiently prolonged even serum or euglobulin from a syphilitic subject may fail to give any precipitation in the syphilitic region. It has further been found that normal serum to which Clupeine sulphate has been added causes precipitation to occur in the syphilitic region and that of mixtures containing euglobulin and clupeine sulphate, pseudoglob-
:ulin and clupeine sulphate , and albumen and clupeine sulphate, respectively, only the first gives rise to precipitation in this region. When the Wassermann reaction is applied to a normal blood serum containing clupeine sulphate in suitable concentration it is found that fixation up to 6 doses of complement may be observ-
:ed and a similar result is obtained by the use of mixt-
:ures of normal euglobulin and clupeine sulphate, but not by albumen and clupeine sulphate. Finally the observation has been made that prelimnary heating at sufficiently high temperature and for a sufficiently

long period, of mixtures of normal serum and clupeine sulphate, alters them in such a way that they fail to fix complement, although they are able to do so after they have been heated at 55°C for periods up to 30 minutes.

We shall now discuss one or two points which arise. Some reference may be made here to the paper by Wolf and Rideal (9) who also examined the effect of Clupeine sulphate upon the precipitation of gum benzoin. These authors obtained the following readings for the precipitation of gum benzoin by a mixture of clupeine and globulin.

Table .

| | 1/100 | 1/200 | 1/400 | 1/800 | 1/1600 | 1/3200 | 1/6400 | 1/12800 | 1/25600 | 1/51200 | 1/102400 | 1/204800. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | + | + | | + | | | | + | |
| Globulin alone. | 0 | 0 | 0 | 0 | 3 | 4 | 3 | 3 | 2 | 1 | 0 | 0 |
| | | | | | | | | | + | + | | |
| " " + Clupeine. | 4 | 3 | 0 | 1 | 4 | 4 | 4 | 3 | 1 | 1 | 0 | 0 |

According to the notation of Wolf and Rideal + indicates slightly greater degree of precipitation than 1.

The dilutions used ~~made~~ by these authors commence at 1/100 and are made in 0.9% saline, also they use a gum benzoin sol protected with saponin. The criterion of positivity laid down by them is that precipitation should occur in the centre zone (that is to say at a concentration of about 1/800 - 1/1600), in excess of that observed with a normal fluid. When clupeine is present they obtain precipitation in the first two tubes but consider that the occurrence of precipitation

in this "prezone" is in no way characteristic of syph-:ilitic sera. It is however apparent that these results are in agreement with those obtained by us when it is recalled that we have found that the characteristic phenomena occur with high concentrations of sera.

Some remarks may be made here regarding a difficulty which occurs in carrying out the precipit-:ation experiments described in the previous sections of the thesis. This lies in the fact that serum contains a certain quantity of inorganic salts, and that there-:fore when dilutions are made with distilled water the concentration of these salts progressively decreases. In consequence the tubes as prepared above contain varying quantities of these inorganic electrolytes and so a varying factor is introduced. For this reason it is possibly desireable to repeat certain of the above experiments, the dilutions being made with a solution prepared so as to contain the same concentration of inorganic salts as the original serum. The main results of the present investigation are not fundamentally effected by this consideration, since care has been taken in the preparation of any of the solutions to be examined, to have present inorganic salts equival-ent to those in serum, and so although the variation in the concentration of salts exists from tube to tube of any particular experiment, yet an exactly similar variation occurs in the experiments with which it is compared.

It will be remembered that it was stated in the experimental section, that the experiments on the effect of preliminary heating of serum on the precipit-:ation phenomena were for the most part carried out with ascitic fluid. It may be mentioned that many of the other precipitation experiments have also been repeated with ascitic fluid, such as for example the experiments with the separate protein fractions, and that substantially similar results have been obtained as with blood serum ~~and with ascitic fluid~~. Ascitic fluid possesses certain advantages for some of the work in that it is relatively poor in protein and when derived from a case of syphilis may give strong reactions in the Wassermann and Sigma tests. Further it is some-:times available in large quantity, and so, adequate amounts of homogenous material may be obtained. We have however as far as possible presented experiments carried out on blood serum since in the fixation experiments only serum was used, but the precipitation results may in general be taken as applying also to ascitic fluid. As already mentioned (page 57) it is of interest to note that the three types of fluid, cerebro spinal fluid, ascitic fluid, and blood serum, containing widely different concentrations of protein, effect in an essentially similar way the precipitation of gum benzoin when the examination is made by the method described.

The general question remains now to be discussed as to what conclusions may be drawn from the above experiments as to the mechanism of the precipitation and fixation phenomena which are observed with sera and other body fluids from a syphilitic individual. The simplest assumption would seem to be that as a result of syphilitic infection, a substance of a protamine-like nature is formed in the body and circulates in the blood, and that the presence of this compound gives rise both to the precipitation and fixation phenomena. We have shown that such an assumption offers a reasonable and possible explanation of what occurs, in as far as the addition of clupeine sulphate to normal serum does in fact cause it to simulate syphilitic serum. On the other hand certain difficulties present themselves. In the first place there is the general question as to whether the precipitation and fixation reactions are both caused by the same factor, or whether two distinct agencies are usually present simultaneously. It is not proposed to discuss at length this difficult and complicated question. It may however be stated that considerable difference of opinion, amongst workers on the subject, exists on this point, and that it appears difficult to obtain conclusive evidence. The experiments of Mackie (10), on the fractionation of syphilitic sera as a

result of which the fixation factor is largely concen-:trated in one fraction , and the precipitation factor in the other,and also the fact that the two reactions do not always run parallel when a number of sera are compared, appeared at first sight to afford strong evidence in favour of the view that there is a separate factor for each type of reaction. On the other hand the close agreement between the results of the Sigma reaction and the Wassermann reaction when they are carried out under suitable conditions is strongly suggestive of the view that they have a common basis. Further,various types of precipitation tests frequently give results not all together consistent with each other, and when tested by the Wassermann reaction, sera may be found which give a positive reaction with one antigen and a negative reaction with another,whilst with other sera, the first antigen gives a negative reaction and the second a positive. The above argument would seem to indicate therefore a multiplicity of substances, one being responsible for each test and having a part-:icular affinity for each antigen. The difficulties in the way of assuming this multiplicity of specific substances appear to be great. In any case it would seem reasonable to suggest a compromise between the two opposite views.

According to this, some one substance would fundamentally be responsible for the fixation and precipitation reactions. This substance may be conceived of as capable of changing the state of the surface of the particles of the colloidal suspension used, whether antigen derived from animal tissue, or a colloidal suspension such as gum benzoin sol. According to the general conceptions suggested above this alteration might consist in the neutralisation, partially or completely of the charge on these particles. When this charge is neutralised, precipitation of the antigen might occur, but it need not necessarily occur; and provided that a sufficient quantity of suitable protective substance is present, it will not occur. On the other hand the same fundamental alteration might facilitate fixation of complement. It appears probable that at a Ph 7 - 8 __the $P_h$ at which the Wassermann reaction is carried out__ complement is negatively charged and so it will be less readily adsorbed on the surface of a colloidal suspension, if the latter is itself negatively charged; but, again, the adsorption of the complement does not necessarily occur as the result of the neutralisation of the charge on the particles. The phenomena taking place at surfaces are very complex, and the protein molecules present, probably have considerable power to alter the

surface tension and modify the surface forces. Thus in the experiments we have described, the presence of a sufficient quantity of euglobulin appears to be necessary in order that clupeine sulphate should effect characteristic fixation of complement in the presence of antigen and the presence of albumen appears to exert an inhibitory action. When serum is fractionated, the necessary conditions requisite for the development of precipitation or fixation may be present in different fractions, and the complicated phenomena which are observed when the various fractions are mixed seem to be consistent with this general point of view.

A further point may be discussed here. It has been long recognised that the changes which occur as the result of syphilitic infection as manifested by the Wassermann Reaction are of a quantitative rather than a qualitative nature. Mackie and Watson (1926)(11) have recently emphasised this point. They have claimed that normal serum mixed with a lipoid antigen possesses in a masked state the power of fixing com-:plement up to six doses (by method B), but that this power is lost if the serum is previously heated to 62°C. This would not seem in any way to be in-:consistent with the point of view presented in the previous paragraph, since protamine like substances might well be present in small amounts in normal sera and we have already shown that their effects

may be marked under certain conditions, as for example in presence of too much albumen. On the contrary it might be agreed that the effects described in this thesis occurring as the result of the addition of clupeine sulphate to normal serum, are due to an unmasking of this normal fixing property as a result of some alteration in the physico-chemical properties of the serum. The fact that the raction disappears if the mixture and clupeine sulphate is submitted to to prelimnary heating at 62°C might be held to indicate that the fixation that manifests itself after the addition of clupeine sulphate is in reality occasioned by a partially labile serum principle, and not directly by thermostable chemical compound, which has been added to the serum.

Clearly however this cannot be held to be an irrestible objection to the above theory, since it is part of the theory set forth on page 95, that the proteins present play a rôle in the fixation phenomena, and alteration in the physical properties of these proteins undoubtedly occurs at 62°C.

In this connection the coagulation which was observed in the experiment described on page 82 may be recalled , since the occurrence of coagulation shows that the proteins had undergone

very considerable changes as the result of being heated for 30 minutes at 62° C. The phenomena dealt with are obviously complex , and definite conclusions at this stage would be premature.

It is of course quite evident that the simulation of syphilitic serum by normal serum containing clupeine sulphate is only partial and in many ways incomplete. It is well to emphasise this point.

The incompleteness of the agreement is more marked, as one would expect in the fixation phenomena, which are highly specific.

The two main points in which disagreement occurs are, (1) that , no matter what concentrations of clupeine sulphate is added , fixation of more than six doses of complement has not been observed, without the occurrence of fixation in some of the tubes in which antigen was absent, whilst strongly positive sera may fix a much larger number of doses , and (2) , that when

large amounts of clupeine are present, anticomplementary results are obtained. These anticomplementary results occur readily, and conditions must be adjusted with some care, if they are to be avoided. Too great emphasis however need not be laid on these difficulties. In the first place, as explained in the ~~previous~~ preceeding paragraph, the factors which influence the fixation phenomena are complex, and it is difficult to ensure that all the secondary factors which may be of some importance are favourable. For instance in syphilis an increase in the percentage of globulin occurs in serum, and the ratio of serum globulin to serum albumen is increased. We have seen that when this ratio is decreased the fixation results obtained by the use of clupeine sulphate become less positive. In the experiments described above in which clupeine sulphate was added to normal serum, the globulin - albumen ratio was not changed but continued to be that of normal serum. This indicates how it is possible for the secondary alterations which occur in serum as the result of syphilitic infection to be of importance in the Wassermann reaction. Further experiments in this direction appear to be desirable.

In the second place it has already been emphasised that clupeine sulphate was chosen for the above experiments because it was the most readily accessible protein of high isoelectric point. There is no suggestion that clupeine itself is responsible for the fixation and precipitation reactions which occur with syphilitic sera. It is of course well known that different protamines are obtained from the milt of different species of fish, and if any substance of this type is really present in syphilitic sera it would almost certainly not be chemically identical with clupeine. Under these circumstances even partial simulation of syphilitic serum by normal serum containing clupeine sulphate is of particular significance. It would be of great interest to repeat many of the above experiments using instead clupeine, which is derived from herring, a protamine obtained from some other fish, or from some quite different source. Some experiments in these directions have already been initiated.

Finally it must be recognised that, so far, no independent evidence has been obtained that syphilitic sera do actually contain a protamine or other protein of high isoelectric point. It is clear that the most rogorous proof would be the isolation of ~~the~~ a substance of this type in greater quantities from syphilitic serum than from normal serum.

The difficulties in the way of separating such a compound from the complicated mixture of proteins present in serum appear to be considerable. It would perhaps be easier to obtain a definite result by the use of cerebro spinal fluid the protein content of which is much lower, but in this case the difficulty of obtaining sufficiently large quantities arises.

We have already made some attempts to obtain positive chemical evidence but so far the results have been somewhat ambiguous, and, because of this, are not presented in the present thesis. Under the circumstances the absence of independant chemical evidence must not be considered an irrestible objection to the general argument advanced above, but on the other hand it is highly desirable that further work in this direction should be carried out.

Appendix i.

Method "A"

The routine method of carrying out the Wassermann test in the Royal College of Physicians Laboratory is as follows:-

For routine examination two types of rack are used a single rack containing 10 tubes and one containing five rows of ten tubes. A 1 in 5 dilution of each serum is made in each tube in the single rack. 0.2c.c. of this dilution is pipetted into each of the corresponding tubes in the second rack. Antigen "A", (0.2c.c.) is added to each tube in the first and third rows, and a similar amount of antigen "B" to each tube in the fifth row. 0.2 c.c of a 0.85% solution of sodium chloride is then added to each tube in the second and fourth rows, and these rows act as controls to those rows to which antigen has been added.

To the first and second rows 2 doses of complement are added,(0.2c.c. to each tube-); a similar amount of 3 doses of complement ~~are~~ is added to each of the tubes in the fourth and fifth rows and of 6 doses to each tube in the third row.

Each tube now contains 0.6c.c of fluid. The tubes are well shaken and placed in the incubator for half an hour at 37°C.

The suspension of red blood cells are now sensitised by the addition of an equal volume of Immune body.
At the end of 3o minutes the tubes are removed from the incubator and 0.2c.c. of sensitised cells is add-:ed to each tube.
The tubes are well shaken and placed in the incubator for 15 minutes at 37°C, then again shaken and incubat-:ion continued for a similar period.
The tubes are removed from the incubator at the end of this period and readings made.

Appendix. ii

Method "B"

The method of carrying out the Wassermann test in the Bacteriological laboratory of the University of Edinburgh is as follows:-

0.05c.c. of the serum to be examined is pipetted into each of 4 tubes.

0.5 c.c. of antigen is then added to each tube.

2 MHD of complement is then added to the first tube,; 4 MHD of complement is added to the second, 8 MHD to third, and 14 MHD to the fourth. The spacing of the doses of complement can of course be altered to suit the requirements of the test.

Serum and antigen controls are included, 0.5 c.c. of 0.85% saline being added instead of antigen in the serum control, and neither serum nor antigen in the antigen control. To both of these controls complement is added, the number of controls set up being determined by the requirements of the test.

The mixtures are well shaken and are incubated for one and a half hours at 37°C and then to each tube 0.5c.c. of the haemolytic system is added. The tubes are shaken and again incubated for one hour when the readings are made.

Appendix iii.

Preparation of colloidal gum benzoin.

10 grams of powdered Sumatra gum benzoin are allowed to remain in contact with 100c.c absolute alcohol for 24 hours at room temperature and then filtered.

0.3c.c. of this alcoholic extract is added with vigorous agitation to 20c.c. distilled water at 55°C and the resulting suspension filtered and cooled.

Appendix iv.

Preparation of Clupeine sulphate.

Soft herring roes, milts, are obtained and ground up in water and the resulting pink white fluid pressed through gauze so that the connective tissue etc. is retained. To the filtered fluid acetic acid is added to cause coagulation.

The fluid is now filtered through a Buchner funnel under negative pressure and the slightly opalescent filtrate rejected. The residue is shaken up in 98% alcohol and brought to the boiling point on the water bath, and then refiltered.

This process is repeated three times, and the final residue is extracted with ether in a Soxhlet to remove fats. The material is now dried at room temperature and stored in a stoppered bottle till required.

100 grams of this material are allowed to stand in contact with 500c.c.1% sulphuric acid and then filtered. This is repeated till all the protamine is extracted. The filtrates are then combined. To this is added three times its volume 98% alcohol. The precipitate is a fine white powder, some of which appears to form a grey-white gummy mass. It is possible to separate these two fractions.

Each fraction should now be worked up separately. The white solid is separated at the filter pump and dissolved in 1 litre distilled water in an evaporating dish. The volume is reduced on the water bath to about 200 c.c. and the fluid poured into a separating funnel. On cooling a yellow oil separates out.

Further purification is resorted to by precipitating by sodium picrate. The clupeine picrate is immediately filtered off shaken up with dilute sulphuric acid and ether until a colourless solution is obtained and then again precipitated with 98% alcohol when a pure white powder separates out.

## REFERENCES.


1. Sachs Georgi. Arb.a.d.Inst.f.Exp.Ther.:Frankfurt-a-Maine.Heft 10,pl. 1920.
2. Dreyer . Lancet 1921. ii. 956.
3. Kahn."Serum Diagnosis by Precipitation". Baltimore 1925.
4. Kermack and Voge. Proc.Roy.Soc.Ed.xlv.1.(10).90.
5. Wright and Kermack:Biochem.J.xvii.4,5.1923.635.
6. Kossel. Zeit.Physiol.Chem.22 . 178. 1896.
7. Mahlo."Chemical Aspects of Immunity" Wells,p,185.
8. Stern. Bio. Zeit. cxliv 1924 and Klin.Woch.2 1923. 145.
9. Wolf and Rideal. J. of Hyg. xxv. 1926.366.
10. Mackie . J. of Hyg. xxi.4.386.1923.
11. Mackie & Watson. J. Hyg. xxv.2. 176. 1926.

*REPRINT FROM THE*

PROCEEDINGS

OF THE

ROYAL SOCIETY OF EDINBURGH.

SESSION 1924–1925.

VOL. XLV—PART I—(No. 10).

# The Action of Salts with Multivalent Cations on Colloidal Solutions of Gold and Gum Benzoin.

By William Ogilvy Kermack and Cecil Innes Bothwell Voge.

EDINBURGH:

PUBLISHED BY ROBERT GRANT & SON, 126 PRINCES STREET, AND WILLIAMS & NORGATE, LTD., 14 HENRIETTA STREET, COVENT GARDEN, LONDON, W.C.2.

MDCCCCXXV.

*Price One Shilling.*

# X.—The Action of Salts with Multivalent Cations on Colloidal Solutions of Gold and Gum Benzoin.

By William Ogilvy Kermack and Cecil Innes Bothwell Voge. From the Research Laboratory of the Royal College of Physicians, Edinburgh. *Communicated by* Dr Alexander Lauder.

(MS. received November 26, 1924. Read December 8, 1924.)

It has been pointed out in a previous paper (Wright and Kermack, 1923), that whereas the precipitation of colloidal gum benzoin by salts containing a uni- or bi-valent cation commences at a given concentration and continues to take place with increasing concentration, in the case of ferric chloride, there occurs with continuously increasing concentrations of this salt, first non-precipitation, second precipitation, then a range of concentrations where no precipitation occurs, and lastly precipitation again. Such a series of changes has been observed with other sols under the action of tervalent ions (*cf.* Burton, 1916). As, however, in a series of dilutions as normally carried out, there occur changes in the $pH$ as well as in the concentration of the ferric chloride, it appeared to be of interest to study the effects of varying both $pH$ and salt concentration and to extend the investigation to various tervalent ions and to other sols. The effects of the following salts were examined: ferric chloride, aluminium chloride, lanthanum chloride, and also calcium chloride and beryllium sulphate. It will be recalled that in the case of aluminium chloride (Wright and Kermack, *loc. cit.*), no zone of precipitation occurred, but that this salt appeared to behave like the salt of a univalent cation. It appears that the difference between its action and that of ferric chloride is merely accidental, and that a similar region of non-precipitation is obtained if the $pH$ is suitably adjusted.

It may be pointed out that these results have a direct bearing on the reaction of amphoteric substances such as gelatin, with colloidal suspensions. It will be seen on comparing, for example, fig. 4 with fig. 2 of the previous paper (Wright and Kermack, *loc. cit.*), that the effect of salts which behave as amphoteric electrolytes on colloidal suspensions is very similar to that of proteins. There exists a region where the amphoteric electrolyte in a colloidal condition is able to confer on the lyophobe negatively charged colloid a positive charge, and the zone between this region of positive charge and the region of normal negative charge is one in which precipitation takes place.

## Experimental.

The colloidal "benzoin" used was prepared as follows:—10 g. of powdered Sumatra gum benzoin were allowed to remain in contact with 100 c.c. of absolute alcohol for twenty-four hours at room temperature and then filtered. 0·3 c.c. of this alcoholic extract was added with vigorous agitation to 20 c.c. of distilled water at 65°, and the resulting suspension was filtered and cooled.

The gold sol was prepared by adding to 100 c.c. of distilled water 1 c.c. of a 1 per cent. solution of photographic gold chloride, 1 c.c. of 0·7 per cent. potassium carbonate (previously dried and ignited), heating to boiling point, and then adding with vigorous shaking, after removal of the flame, 1 c.c. of a 1 per cent. solution of neutralised 40 per cent. formalin. If the glass vessels used are clean, and the distilled water pure, no difficulty is experienced in obtaining bright red sols with practically no shimmer. Occasionally failure has been caused by contamination of the distilled water. The glassware used is thoroughly cleansed by immersion in sulphuric acid-bichromate mixture, followed by thorough washing.

The order of experimentation is to prepare a series of dilutions of the salt, and also a series of dilutions of alkali or acid. A block of test-tubes is then arranged in rows and columns, each column corresponding to one of the concentrations of the salt solutions, and each row to one of the dilutions of acid (or alkali). 1 c.c. of salt solution is then added to each test-tube in the corresponding column, and 1 c.c. of acid (or alkali) to each test-tube in the corresponding row, so that each test-tube contains 2 c.c. of water containing salt and acid (or alkali) of a definite concentration. 2 c.c. of the gold sol or "benzoin" sol are then added to each test-tube, the mixture shaken thoroughly and allowed to stand overnight. Readings are then made according to the following convention. "Benzoin" sol:—(0) no precipitation; (1) slight precipitation at bottom of tube; (2) partial precipitation; (3) almost complete precipitation; (4) complete precipitation. Gold sol:—In general two types of precipitation are observed, with the formation of a red or of a purplish-blue precipitate respectively. In either case (5) denotes complete precipitation, with a clear supernatant liquid; (0) denotes no change; whilst (1), (2), (3), and (4) denote intermediate degrees of precipitation, similar to the notation already described for the "benzoin" sol. In the case of those tubes in which the precipitate was red, note was made of this fact. The degree of precipitation having been observed, the $pH$ of the supernatant fluid in those "precipitated" tubes which appeared to be important was determined colorimetrically.

TABLE I.—ALUMINIUM CHLORIDE AND COLLOIDAL GOLD.

| Conc. of $AlCl_3$ :— | M/20. | M/40. | M/80. | M/160. | M/320. | M/640. | M/1280. | M/2560. | M/5120. | M/10240. | | M/40960. | | M/163840. | | M/655360. | | M/2621440. | $H_2O$. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Concentration of HCl. | | | | | | | | | | | | | | | | | | | |
| N/25 | **5** 3·6 | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... |
| N/50 | **5** 4·4 | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** 3·0 | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **4** ... | **5** ... | **4** 2·0 |
| N/100 | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **3** 2·4 | **3** 2·3 | **5** 2·2 | **3** ... |
| N/200 | **5** ... | **5** ... | **5** ... | **4** ... | **4** 3·6 | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **4** ... | **5** 3·2 | **2** ... | **1** ... | **0** ... |
| N/400 | **5** ... | **5** ... | **3** 4·4 | **2** ... | **0** ... | **2** ... | **4** 4·4 | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **4** ... | **5** 3·4 | **0** ... | **0** ... | **0** ... |
| N/800 | **5** ... | **5** ... | **3** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **2** 5·4 | **3** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **5** 4·2 | **2** ... | **1** ... | **0** ... |
| N/1600 | **5** ... | **4** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** 5·8 | **1** ... | **3** 4·4 | **5** 4·4 | **5** ... | **5** ... | **5** 4·1 | **5** 4·2 | **5** 4·0 | **0** ... |
| N/3200 | **5** ... | **3** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **5** 4·0 | **5** ... | **5** ... | **5** ... | **5** ... | **5** ... | **0** ... |
| N/6400 | **5** ... | **3** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **1** ... | **1** ... | **3** 5·0 | **5** 5·2 | **5** ... | **5** ... | **5** 5·6 | **2** 5·6 | **0** ... |
| N/12800 | **5** ... | **3** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** 6·4 | **0** ... | **1** ... | **2** 6·2 | **4** 5·4 | **5** ... | **5** ... | **5** 5·8 | **5** 6·0 | **0** ... |
| N/25600 | **5** ... | **5** ... | **1** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **1** ... | **0** ... | **0** 6·4 | **1** 6·2 | **2** 6·2 | **4** 5·8 | **5** ... | **4** ... | **4** ... | **5** 6·4 | **0** ... |
| $H_2O$ | **5** ... | **4** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** 5·2 | **0** 5·4 | **0** 6·8 | **0** 6·6 | **1** 6·2 | **1** 6·2 | **3** ... | **5** 6·2 | **5** ... | **5** ... | **5** ... | **0** ... |
| Concentration of NaOH. | | | | | | | | | | | | | | | | | | | |
| N/25600 | **5** ... | **4** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** 5·2 | **0** 5·4 | **0** 5·8 | **0** 6·6 | **0** 6·6 | **0** 6·4 | **1** ... | **3** 6·4 | **4** 6·6 | **5** 6·8 | **5** 7·0 | **0** ... |
| N/12800 | **5** ... | **3** .. | **0** ... | **0** ... | **0** ... | **0** ... | **0** .. | **0** ... | **0** ... | **0** ... | **0** ... | **0** ... | **1** ... | **1** 6·8 | **5** ... | **5** 6·8 | ... ... | ... ... | **0** ... |
| N/6400 | **5** ... | **3** 6·4 | **0** ... | **0** ... | **0** ... | **0** 5·0 | **0** ... | **0** ... | **0** ... | **0** ... | **0** 6·8 | **0** 7·0 | **4** 6·6 | **5** ... | **5** ... | ... ... | ... ... | ... ... | **0** ... |
| N/3200 | **5** ... | **2** ... | **0** ... | **0** ... | **0** ... | **0** ... | **0** 5·0 | **0** 5·4 | **0** 6·4 | **0** 6·5 | **5** 7·2 | **3** ... | **3** 7·4 | **1** ... | **0** ... | ... ... | ... ... | ... ... | **0** ... |
| N/1600 | **5** ... | **1** ... | **0** ... | **0** ... | **0** ... | **0** 5·0 | **0** 5·4 | **0** 6·4 | **0** 6·6 | **5** 7·2 | **4** 7·4 | **0** ... | **0** ... | **0** ... | **0** ... | ... ... | ... ... | ... ... | **0** ... |
| N/800 | **5** ... | **1** ... | **0** ... | **0** ... | **0** 5·0 | **0** 5·2 | **0** 6·4 | **0** 6·6 | **5** 7·4 | **4** 7·6 | **0** ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | **0** ... |
| N/400 | **5** ... | **0** ... | **0** ... | **0** 4·8 | **0** 5·0 | **0** 5·2 | **0** 6·0 | **5** 8·2 | **5** 8·4 | **0** ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | **0** ... |
| N/200 | **5** ... | **0** ... | **0** ... | **0** 5·0 | **0** 5·2 | **5** 8·2 | **5** 8·8 | **2** ... | **0** ... | **0** ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | **0** ... |
| N/100 | **3** ... | **0** ... | **0** ... | **0** 5·0 | **5** 8·8 | **5** ... | **4** 9·8 | **4** ... | **0** ... | **0** ... | ... ... | ... .. | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | **0** ... |
| N/50 | **5** ... | **4** ... | **3** 4·2 | **5** 9·2 | **5** ... | **5** ... | **5** ... | **3** 10·0 | **2** ... | **0** ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | **0** ... |
| N/25 | **0** 4·4 | **1** 4·4 | **5** 9·6 | **5** ... | **5** 9·8 | **2** ... | **1** ... | **0** ... | ... ... | ... ... | ... ... | .. ... | .. ... | ... ... | ... ... | ... ... | ... ... | ... ... | **0** ... |
| N/12·5 | **5** 10·2 | **5** ... | **5** ... | **4** . | **3** 10·2 | **2** ... | **0** ... | ... ... | ... ... | ... ... | ... .. | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | **0** ... |
| N/6·25 | **5** 12·8 | **5** ... | **4** ... | **3** ... | **1** ... | **0** ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | ... ... | **0** ... |

(The numerals in heavy type denote the degree of precipitation and the other numerals the *pH* values.)

The results of an experiment with $AlCl_3$ and gold sol are shown in Table I, and to represent the important features more clearly the results have been transferred to fig. 8, the shaded regions representing precipitation, *i.e.* regions in which corresponding tubes show a precipitate of either 3, 4, or 5. The results in other cases are represented by figs. 1–7 and 9, the figures in each case being prepared from a table similar in principle to Table I. The actual tables we have not considered it necessary to submit, as practically all the important information is contained in the figures.

It may be noted that the important feature is the relative position of the various regions in the figure. Not much emphasis is to be laid on the exact value of the concentrations, etc., at which precipitation takes place, particularly as very slight changes in the preparation of the colloid in its concentration, etc., cause variations in the results, but the general relation of the negatively charged, the positively charged, and the precipitated regions are constant, but as will be seen vary from salt to salt. It may be noted that the limits at high concentrations of salt are vague as the $pH$ varies there in large steps.

## Discussion.

Two very marked differences are at once apparent between the effect of bivalent and that of tervalent cations. Firstly, the tervalent ions precipitate in very much lower concentrations than the bivalent ions. For instance, taking the amount of salt required to precipitate "benzoin" sol at $pH$ 5 for purposes of comparison, there is required in the case of calcium chloride, M/80; of beryllium sulphate, M/80; of lanthanum chloride, M/1280; of ferric chloride, M/10240; and of aluminium chloride, M/20480. Secondly, it is seen that with calcium and beryllium there is no positively charged region. With iron, aluminium, and lanthanum there is a region in which the gold or benzoin is positively charged. The result of this is the appearance with those salts of a zone phenomenon characteristic apparently of tervalent ions.

If we now restrict ourselves to the tervalent cations it would appear that the action of their salts in producing precipitation is related to their degree of hydrolysis. For example, at a concentration of M/160, ferric chloride precipitates "benzoin" at $pH$ 2·7 approximately, but not at a slightly higher $pH$, aluminium chloride at $pH$ 3·8, and lanthanum chloride at $pH$ 5·6. For gold sol, which, in general, is more easily precipitated, the corresponding figures are 3, 3·8, and 6. The following figures showing the

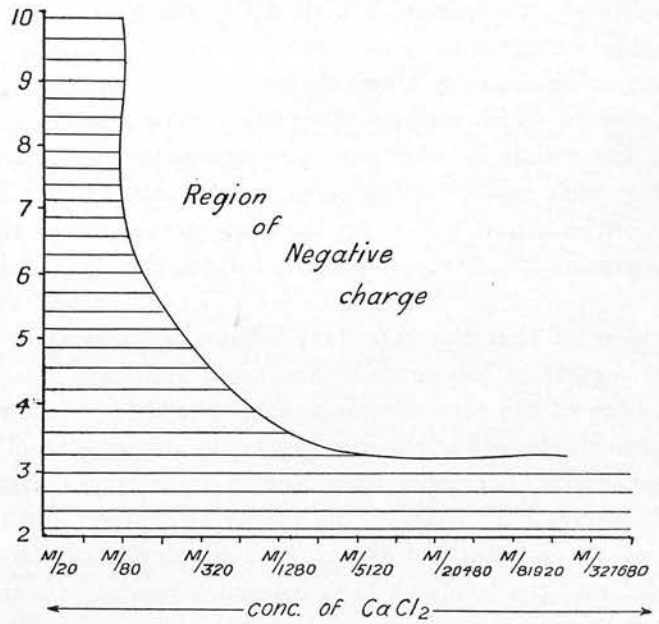



FIG. 1.—$CaCl_2$ and Colloidal Benzoin.

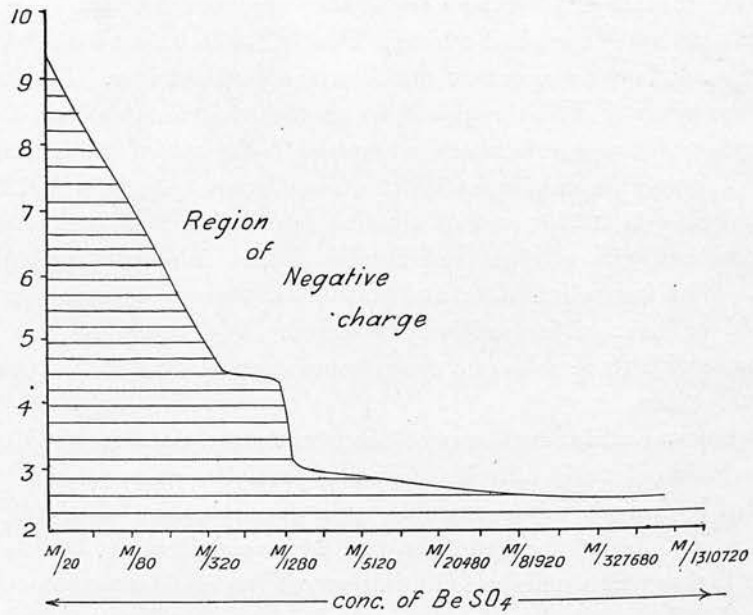



FIG. 2.—$BeSO_4$ and Colloidal Benzoin.

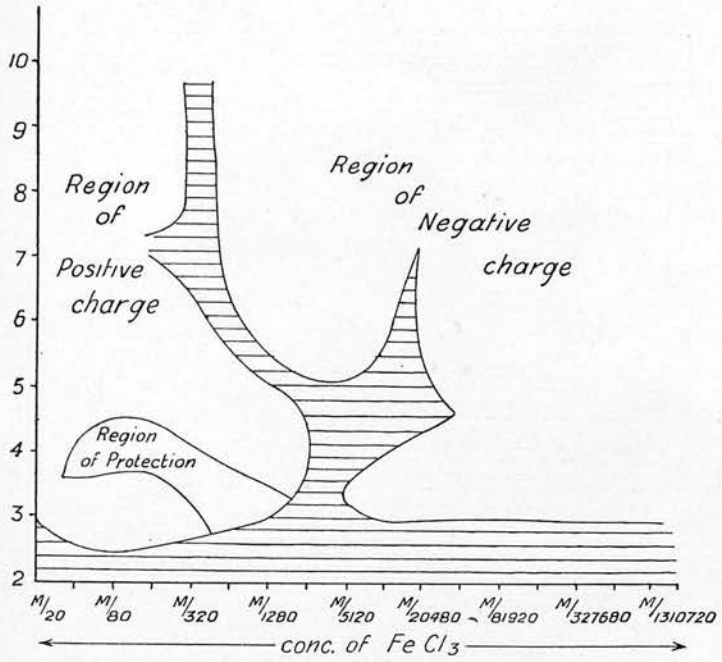



FIG. 3.—$FeCl_3$ and Colloidal Benzoin.

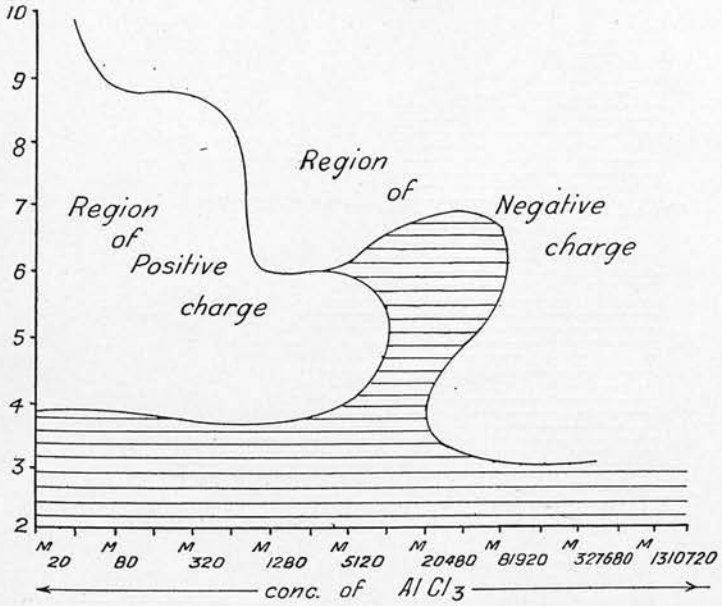



FIG. 4.—$AlCl_3$ and Colloidal Benzoin.

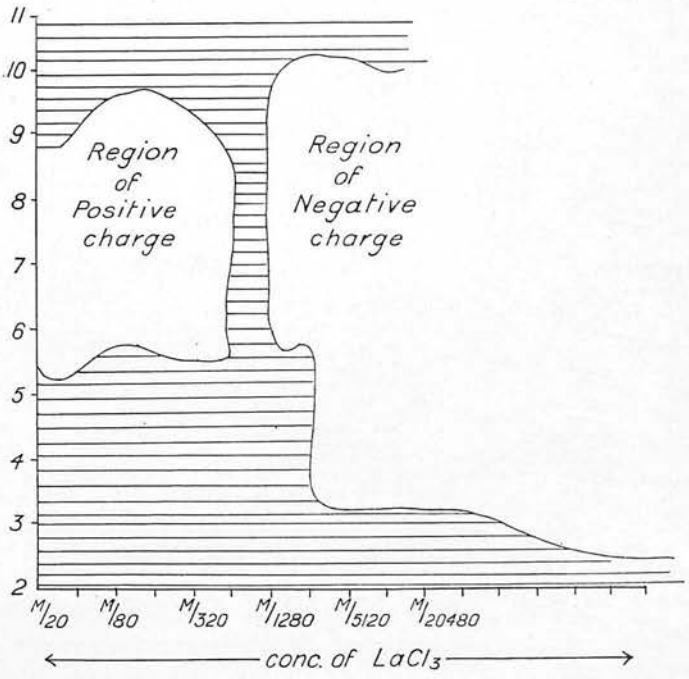



FIG. 5.—$LaCl_3$ and Colloidal Benzoin.

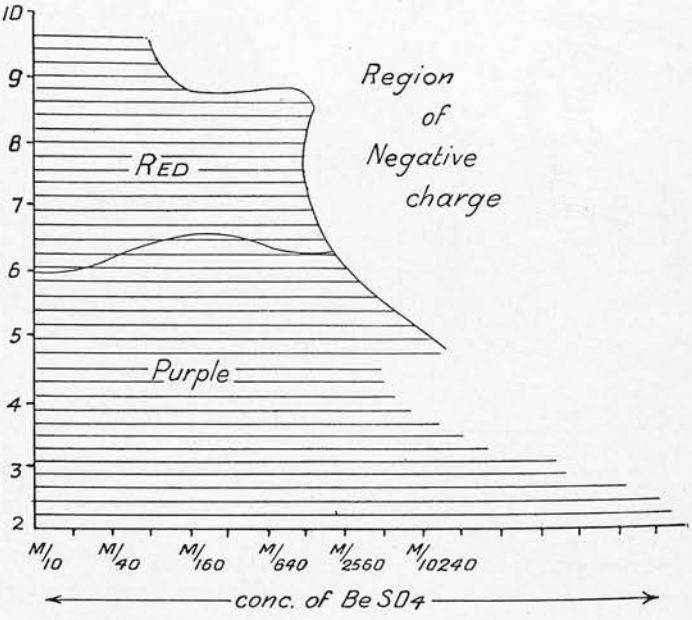



FIG. 6.—$BeSO_4$ and Colloidal Gold.

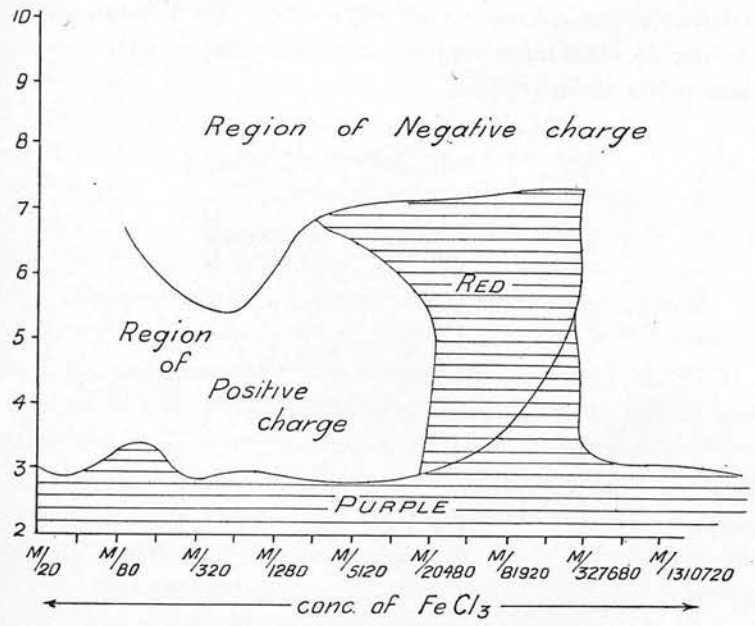



FIG. 7.—$FeCl_3$ and Colloidal Gold.

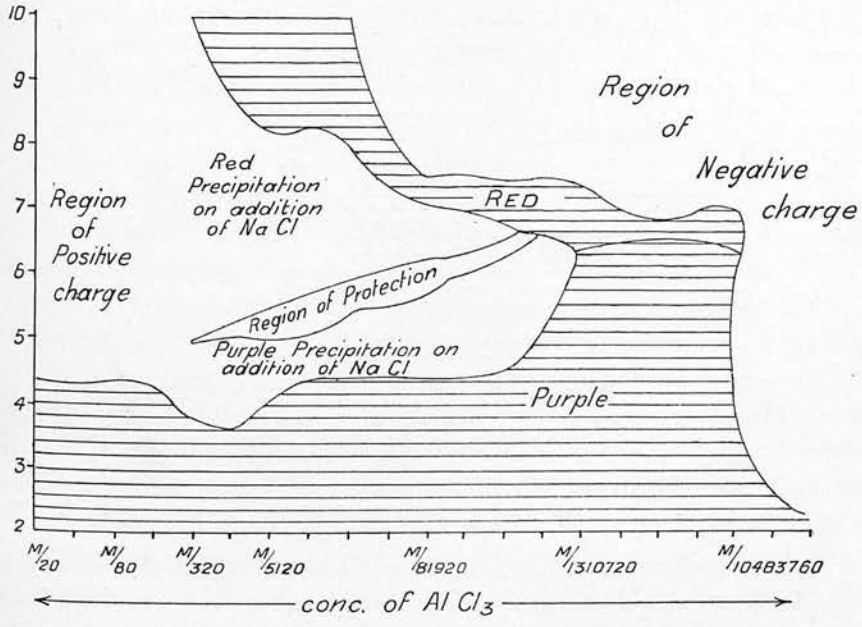



FIG. 8.—$AlCl_3$ and Colloidal Gold.

relation between the degree of hydrolysis and the dilution in the cases of ferric chloride, aluminium chloride, and lanthanum chloride are extracted from Landolt-Börnstein (1923):—

| Salt. | Dilution. | Hydrolysis. |
|---|---|---|
| $FeCl_3$ | 33·34 l | 37 per cent. |
| „ | 666·7 l | 91 per cent. |
| $AlCl_3$ | 1024·0 l | 4·5 per cent. |
| $LaCl_3$ | 32·0 l | 0·00672 per cent. |

The salt hydrolysed in greater degree exerts its power to confer a positive charge on the colloidal particles in more acid solution than does that hydrolysed in a less degree. It would seem, therefore, that this conferring of a

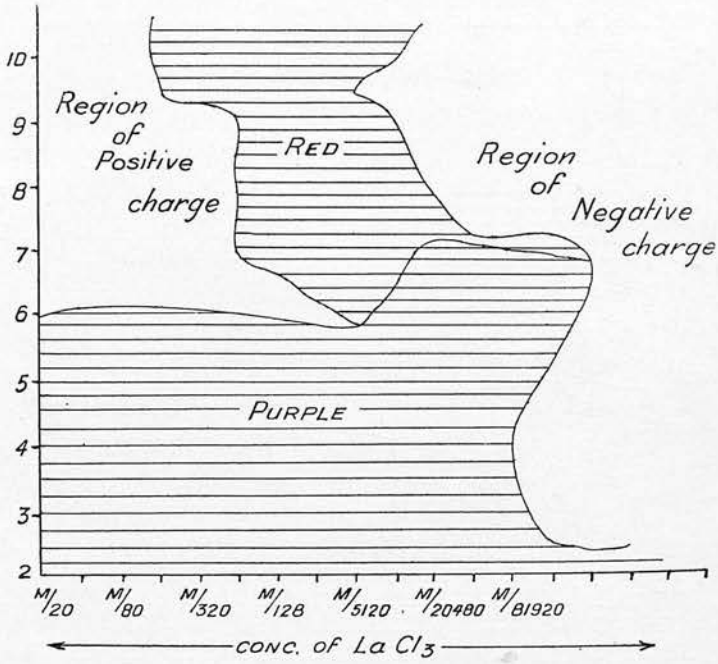



FIG. 9.—$LaCl_3$ and Colloidal Gold.

positive charge is related to the formation of hydroxide. In the case of ferric chloride, hydroxide is formed in appreciable quantities in acid solution ($pH$ 3). In the case of lanthanum chloride, hydroxide is formed only when the reaction is very near the neutral point $pH$ 7, or on its alkaline side.

It would appear, however, that the presence of metallic hydroxide is not alone sufficient to account for the phenomena observed. If this were the case we would expect that on making the solutions more alkaline, the positive charge would remain, and in fact it might be expected to increase. However, considering solutions containing a sufficiently low concentration of salt, there is encountered, after leaving the positively charged region, first of all a zone of precipitation; and then in the case of aluminium or iron, a region where no precipitation occurs, and where the charge is negative. In the case of lanthanum this region is obscured as the insoluble lanthanum hydroxide is precipitated whenever the solution becomes alkaline, thus masking the state of the colloidal gold or "benzoin." It would appear, therefore, that a mere increase in the amount of hydroxide does not ensure stability of the colloidal solution, but that a decrease in stability may actually accompany this. As the amount of hydroxide increases, and with it the $pH$, the number of metallic ions will decrease. The decrease of stability would be accounted for if a certain number of metallic ions were necessary in order that stability should be maintained. The two conditions, then, under which salts of iron, aluminium or lanthanum confer a positive charge on the colloidal particles would appear to be (1) the presence of metallic hydroxide, and (2) the presence of an adequate number of positively charged metallic ions. It is possible that under these conditions a complex between the colloid and the hydroxide is formed, which is associated with a positively charged tervalent ion. The latter confers on the complex a positive charge. This would be analogous to what takes place in the formation of colloidal iron hydroxide, where the presence of the ferric ion would appear to be necessary to render the particles of ferric hydroxide positive, and so stabilise them. These complexes of hydroxide plus ion may be similar in nature to the complexes postulated by Pauli (1920), who suggests that in the case of colloidal iron, for example, a complex $x[Fe(OH)_3] . y[Fe\text{-}An.]$ exists, where An. denotes the anion. Dissociation of the anion from this complex would give rise to a product containing hydroxide associated with positively charged ions similar to that suggested above. This view that a complex is formed between the colloidal particles and the metallic hydroxide is borne out by the fact that in the positively charged region the particles in suspension are larger than in the negatively charged region, as seen under ultramicroscopic observation, and that in the case of experiments with the gold sol, two types of precipitate were noted. In the more acid regions, as will be seen from the figures, the precipitates were purple, in the more alkaline regions red. In the case of aluminium the change takes place about $pH$ 6, in the cases

of iron and lanthanum between *pH* 3 and *pH* 5, and between *pH* 6 and *pH* 7 respectively. Again, these figures correspond to the order of the degree of hydrolysis of the salts. This change which occurs in the more dilute solutions of salt is seen to correspond to the zone of non-precipitation in the more concentrated solutions, which in fact separates the purple from the red precipitates in these regions.

It is of great interest to compare with these results those obtained, using salts with bivalent cations. Here there exists no region in which the charge is positive. Even in the case of beryllium, which in certain respects resembles aluminium more than lanthanum does, and also more than it itself resembles calcium, the resulting type of figure is essentially similar to that obtained with calcium. If our views of the explanation of these phenomena in the case of tervalent ions are correct, then either no association of hydroxide and colloid is possible in the case of bivalent ions, or else there occurs no stabilising association of the free cations with such a complex. It would appear to be quite probable that the second hypothesis is true, for instance it is possible by using an alkaline solution of beryllium sulphate to obtain a precipitation of gold, fig. 6. The explanation may be that tervalent ions are associated with a more powerful atomic field of force, and can form such associated complexes more easily than bivalent ions and with much greater ease than univalent ions. A similar cause may be responsible for the great precipitating power of tervalent ions, as the more powerful the atomic field, the more readily would such ions become adsorbed on surfaces, and hence reduce the charge of colloidal particles. It is well known that amongst univalent ions, those which can form complexes, *e.g.* silver, precipitate more readily than, say, the alkali metals.

In some instances, an equal volume of 2 per cent. sodium chloride solution was added to the non-precipitated positively charged colloidal solutions in order to ascertain whether or not they were protected. Slight protection was evident in the case of gold sol and aluminium chloride, and it is of interest to note that the precipitates on either side of this region were red and purple respectively (see fig. 8). A similar addition of sodium chloride was made in the case of "benzoin" sol and ferric chloride (see fig. 3), the only other combination investigated in this respect. According to our views this would be the region where the highest number of positively charged metallic ions exist simultaneously with sufficient hydroxide to form a surface layer. On the more acid side, there is not enough hydroxide to form such a surface layer, as the gold when precipitated separates in the blue condition, whilst on the more alkaline side the number of metallic ions will presumably decrease.

In conclusion, it will be observed that these solutions of amphoteric molecules derived from metallic salts bear considerable resemblance to solutions of gelatin and of similar proteins in their action on lyophobe colloids. In both cases there is a group possessing considerable affinity for water. In one case it is a polypeptide containing many—NHCO—groups, in the other case a trihydroxy derivative of a metal. Both molecules can acquire an electrical charge, in the one case by dissociation of one of its terminal carboxyl groups, or by the addition of a charged hydrogen to a terminal amino group, and in the other case probably by association with a charged ion.

The effect of all varieties of ions and molecules in modifying the properties of interfacial surfaces is of the greatest importance in the interpretation of the most diverse phenomena of colloidal chemistry, and the elucidation of the mechanism of such effects will undoubtedly prove of the greatest value for the understanding more particularly of biological colloid reactions.

## Conclusions.

(1) Salts of tervalent cations are able to confer, under appropriate conditions of concentration and $pH$, a positive charge on the particles of a negatively charged colloidal sol.

(2) Separating the region where the sol particles are positively charged from the region where they are negatively charged is a zone of precipitation.

(3) These zones are closely related to the degrees of hydrolysis of the salts.

(4) The zone of positive charge may be due to the existence of a molecule of metallic hydroxide which is able to establish itself on the surface between the sol particles and water, and to associate itself with a metallic ion.

(5) No such phenomena have been observed with bivalent ions.

---

## References.

Burton, 1916, *The Physical Properties of Colloidal Solutions*, Longmans, Green & Co., p. 151.

Landolt-Börnstein, 1923, *Physikalisch-Chemische Tabellen*, 5th edition, vol. ii, Springer, Berlin, pp. 1165-1170.

Pauli, 1920, *Report of Faraday Society on the Physics and Chemistry of Colloids*, pp. 14–16.

Wright and Kermack, 1923, *Biochem. Journ.*, **17**, 635.

(*Issued separately April* 29, 1925.)